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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Several approaches toward the development of a bioluminescent immunoassay for TNT have been examined. Three basic types of immunoassays have been studied: (1) Homogeneous Bioluminescent Assay, (2) Heterogeneous Bioluminescent Assay, and (3) Amplified Heterogeneous Bioluminescent Assay. The homogeneous immunoassay involves preparing a TNP-luciferase conjugate which upon binding to anti-TNP antibody exhibits either a significant increase or decrease in luciferase activity, i.e., light production. A large change in light production was not observed and this approach was not pursued further.

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The heterogeneous immunoassay utilizes an antibody to TNP which has been covalently bound to a solid phase, i.e., Sepharose. The free TNT and TNP-luciferase compete for binding sites on the immobilized antibody and the bound luciferase-TNP is measured by light production. There is an inverse relationship between the amount of free TNT in the sample and the amount of TNP-luciferase bound. Using this procedure it was possible to detect 50 femto-moles of free TNT.

The amplified heterogeneous assay is in principle the same as that described for the heterogeneous assay except the TNP is linked to glucose-6-phosphate dehydrogenase rather than luciferase. This enzyme has a large turnover number and therefore one molecule of glucose-6-phosphate dehydrogenase can produce 70,000 molecules of product per minute. This results in a significant amplification step. Using this procedure it was shown that 10 attomoles (10×10^{-18} moles) of TNT could be assayed.

The luminescent enzymes used in this assay have been immobilized and incorporated into a flow cell with an automated sampling device; these enzymes are quite stable and reuseable for repetitive assays.

Various other assays have been evaluated for sensitivity and reproducibility. These are discussed in detail.

Future work will be directed toward increasing the speed of the assay and the possibility of full automation.

DETECTION OF LOW LEVELS OF TNT USING

IMMUNOLOGIC AND BIOLUMINESCENT

TECHNIQUES

FINAL TECHNICAL REPORT

by

Marlene DeLuca
Daniel Vellom

December, 1982

U.S. Army Mobility Equipment
Research and Development Command

U.C.S.D.

Contract No. DAAK 70-79-C-0174

Table of Contents

I.   GENERAL INTRODUCTION

The final report details the work completed between October 1979 through September 1982 on the Contract No. DAAK 70-79-C-0174 entitled "Detection of Low Levels of TNT Using Immunologic and Bioluminescent Techniques."

The specific aim of the research was to develop a bioluminescent immunoassay for the detection of low levels of TNT. The principles involved are essentially the same as for a radioimmunoassay (RIA), i.e., the competition of binding of the antigen of interest (TNT) with a labelled TNT-X for antibody. The major difference from the RIA is that a luciferase or other enzyme is the label on the TNT rather than a radioactive isotope. These assays can be broadly classified as enzyme immunoassays. If the enzyme used as a label is a luciferase, a more appropriate designation is a bioluminescent immunoassay (BIA) (Fig. 1). In this case the end product which is measured is light (hv) not radioactivity. Such assays are potentially rapid and sensitive and require relatively simple equipment for measuring light produced. This new technique was developed in the course of this effort. It permits detection of 10^{-17} moles of TNT.

The first reports of the development of a bioluminescent immunoassay were a direct result of the work performed in this laboratory as a part of this contract. These will be discussed in some detail in later sections.

II.   BACKGROUND AND SUMMARY OF ACCOMPLISHMENTS DURING FIRST HALF OF THE CONTRACT PERIOD

A technical interim report was submitted in July 1981 and therefore we will only summarize the accomplishments for the first half of the contract here.

Prior to this summary we will briefly describe the enzymes utilized in these studies.

a.   Firefly Luciferase

This enzyme is purified and crystallized from firefly lanterns as described previously (1). The enzyme catalyzes the following reactions:

(a) $LH_2 + ATP + E \xrightleftharpoons{Mg^{++}} E \cdot LH_2 \cdot AMP + PPi$

(b) $E \cdot LH_2 \cdot AMP + O_2 \longrightarrow E + P + CO_2 + AMP + \underline{hv}$

2
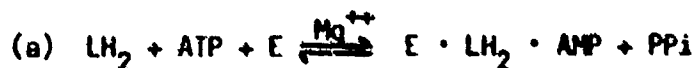
Figure I. General Scheme for Bioluminescent Immunoassay

(1) The immobilized antibody is allowed to incubate with TNT and TNP-luciferase for approximately 2 hours.

(2) The Sepharose-antibody is then washed extensively to remove any non-specifically bound TNT or conjugate.

(3) The bound luciferase is assayed by light production. The amount of bound TNP-luciferase is inversely proportional to the amount of TNT added.

Sepharose - Ab

1. Incubate

TNP-Luciferase

TNT

2. Wash Sepharose

3. Assay Bound Luciferase

LIGHT
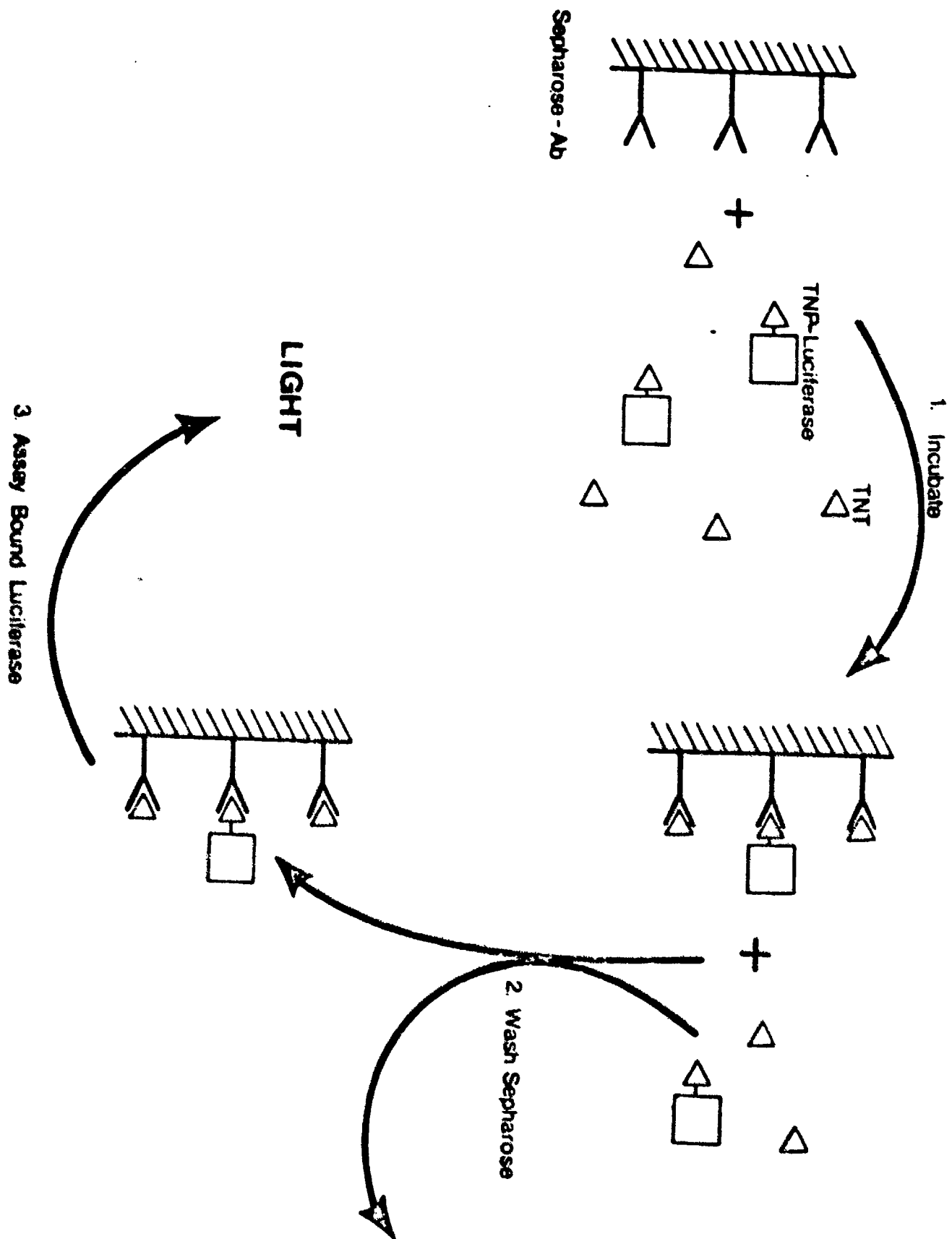
The first reaction is the activation of luciferin (LH_2) to form an enzyme bound luciferyl-adenylate (LH_2-AMP). In the presence of O_2 this enzyme bound intermediate undergoes an irreversible oxidative decarboxylation to produce decarboxy luciferin (P), CO_2, AMP and light (hv). The light emitted is yellow-green, peak at 560 nm, and the quantum yield with respect to luciferin is 0.88 (2). It is a very efficient conversion of chemical energy to light. With the instruments presently available in our laboratory it is possible to detect approximately 50 femtomoles of the enzyme.

The purified enzyme is stable indefinitely when stored in 10% ammonium sulfate at 4°C or can be lyophilized in the presence of BSA and as such has an indefinite shelf-life.

It contains two cysteine residues which are at or near the active site (3). Modification of these cysteines results in a loss of catalytic activity. However, these two cysteines can be protected from modification by the presence of substrates. The enzyme contains about 60 lysine residues. In the presence of ATP-Mg^{++}, up to 15 of these can be succinylated with no loss of apparent activity. Therefore, the possibility of covalently attaching an antigen (TNP) to these lysines is a viable option.

b. Bacterial Luciferase and Oxidoreductase

Bacterial luciferase has been purified and characterized from several species of luminous salt water bacteria (4). While there are some minor differences, in general, the enzyme is an αβ heterodimer with a molecular weight of about 80,000 daltons. It catalyzes the following reaction:

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCOOH + H_2O + hv$$

This is the oxidation of reduced flavin mononucleotide in the presence of a long chain aldehyde with production of FMN, long chain acid and light. The

light is blue with a maximum around 460 nm. The quantum yield of this reaction has been reported to be 0.1 - 0.2.

When the enzyme is assayed by injection of $FMNH_2$ into a solution containing aldehyde and buffer, there is a rapid flash of light followed by a decay (Fig. 2). This is due to the fact that $FMNH_2$ is rapidly autooxidized ($t_{\frac{1}{2}} \sim 3$ sec) and therefore the enzyme essentially turns over only once.

The luminescent bacteria also contain an NAD(P)H:FMN oxidoreductase which catalyzes the following reaction:

$$NAD(P)H + H^+ + FMN \longrightarrow NAD(P)^+ + FMNH_2$$

This enzyme catalyzes the NAD(P)H dependent reduction of FMN. This enzyme has been purified in our laboratory and can be used in a coupled assay with luciferase as shown:

$$NAD(P)H + FMN + H^+ \longrightarrow NADP^+ + FMNH_2$$

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCOOH + h\nu$$
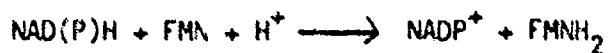
In this assay if NAD(P)H is the limiting substrate then light production will be directly proportional to the concentration of [NAD(P)H]. We have co-immobilized the oxidoreductase and luciferase onto Sepharose 4B and using this preparation we can accurately measure 50 femtomoles of NAD(P)H.

c. Glucose-6-Phosphate Dehydrogenase (G-6-PDH)

This enzyme from Leuconostoc mesenteroides (available commercially) will utilize either NAD or NADP and catalyzes the following reaction:

$$D\text{-Glucose-6-phosphate} + NAD(P)^+ \longrightarrow$$

$$D\text{-Glucose-}\delta\text{-lactone-6-phosphate} + NAD(P)H + H^+$$
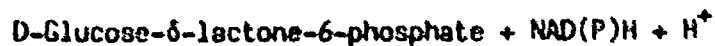
The enzyme has a molecular weight of 104,000 daltons and contains no cysteine or cystines. It has a turnover number of 70,000 (nmoles NADH/nmole enzyme/min). This enzyme, since it produces NAD(P)H, can be coupled to the bacterial

Figure II. Time course of light emission by bacterial luciferase following injection of $FMNH_2$ into a solution containing buffer, decanal and enzyme.
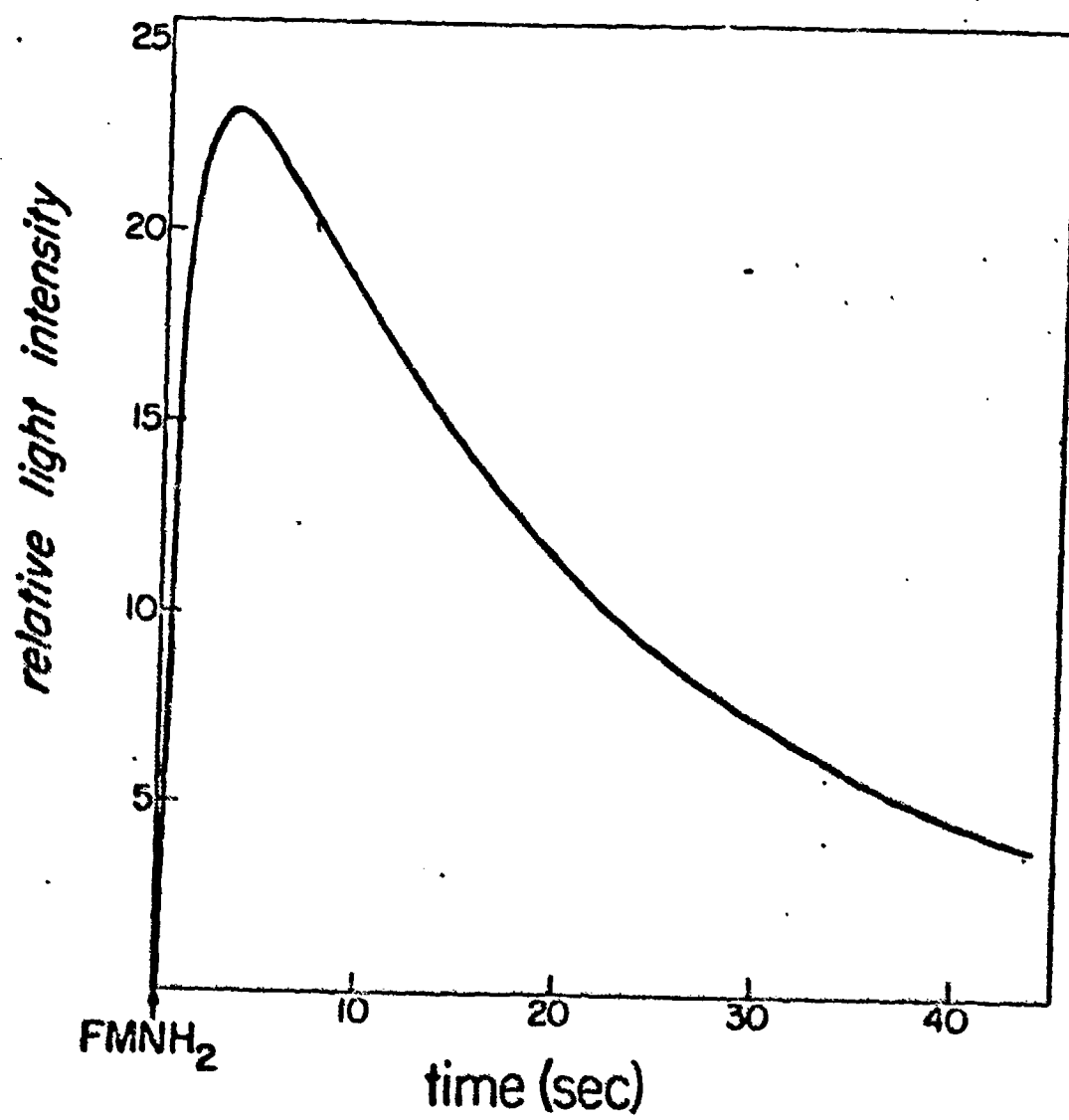
oxidoreductase and luciferase according to the following reactions:

$$G\text{-}6\text{-}P + NAD(P)^+ \longrightarrow 6PGA + NAD(P)H + H^+$$

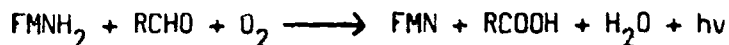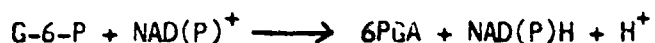$$NAD(P)H + H^+ + FMN \longrightarrow NAD(P)^+ + FMNH_2$$

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCOOH + H_2O + hv$$

If the G-6-PDH is the limiting component in the system, then under defined conditions the amount of NAD(P)H produced will be directly proportional to the G-6-PDH and this can be readily quantitated with the immobilized oxidoreductase and luciferase. The relatively large ~urnover of this enzyme allows an amplification step to be introduced as described below.

We have utilized all of these enzymes in our initial attempts to develop a sensitive bioluminescent immunoassay for TNT. Before discussing these assays a description of the methods used for the isolation of an antibody for TNT as well as the methods of preparation of luciferase TNP and G6PDH-TNA conjugates will be given.

d.   Antibody Production and Isolation

Synthesis of Antigen

The BSA-TNP antigen for injection into the goats and rabbits was synthesized by adding equal volumes of 12 mg/ml BSA in 0.2 M Borate pH 9.2 and 12 mg/ml TNBS in H_2O. After incubating at 40°C for eight hours, the mixture is extensively dialyzed against 0.1 M PO_4 pH 8.0. The reaction mixture is then passed through a G-25 Sephadex column to remove any remaining free TNP from the BSA-TNP. The column is eluted with 0.1 M PO_4 pH 7.8. The degree of derivatization is determined by OD_{350} where the A_{TNP} = 15,400.

Procedure for Antibody Production by Lymph Node Injection

- Inject 0.2 ml of a 2.5% solution of Evans Blue subcutaneously between toes of each hind foot of a rabbit 1 hour previous to surgery.

     2.5% Evans Blue in physiological saline.

     Filter.

     Add merthiolate and store at 4° C.

- To prepare animal for surgery, inject 1.5 ml per 6-8 lbs. body weight Diobutal into ear vein. Shave popliteal area of both hind legs.

- Expose lymph nodes.

- Inject total of 0.1 ml volume into each lymph node. To prepare protein, use approximately 20 μgm in .1 ml and add equal volume of Freund's adjuvant (complete). Mix thoroughly using 1 ml syringe and 20 gage needle. Inject through 25 gauge needle.

- Four weeks later. Inject approximately 100 μgm of protein mixed with equal volume of Freund's adjuvant into 3 sites; intramuscularly into each hind leg and subcutaneously into neck. (.2 ml protein solution I.V. optional).

- Bleed after 10 days.

Procedure for Ammonium Sulfate Fractionation of the IgG & IgM from Blood Serum

1.   Spin 7,000 rpm (SS-34 head) for 10 minutes to spin out R.B.C.
     Discard pellet

2.   25% A.S. fraction (144 gm/1) (A.S. = Ammonium Sulfate)

3.   Spin 7,000 rpm (10 min.)

4.   Discard pellet

5.   37% A.S. fraction (77 gm/1)

6.   Spin 7,000 rpm (10 min.)

7.   Dissolve pellet (IgG + IgM) in H_2O (min. vol.)

8.   Dialyze overnight against 1 l H_2O (4°C)

9. Spin 7,000 rpm (10 min.)

10. Take pellet (IgM) up in 0.1 M PO_4 pH 7.8/0.15 M NaCl

11. Dialyze supernatant fraction (IgG) against 0.1 M PO_4 pH 7.8/0.15 M NaCl

e. <u>Preparation of Luciferase and G6PDH Conjugates</u>

The optimal conditions for preparing TNP-luciferase conjugates were as follows:

- Mix 50 nmoles TNBS and 10 nmoles luciferase in 0.1 M PO_4 pH 7.8 with 2 μmoles of ATP and 1 μmole Mg^{+2}.

- Incubate for 12 hours at 4°C.

- Extensively dialyze against 0.1 M PO_4 pH 7.8, 10^{-4} M DTT and 10^{-3} M EDTA.

- Pass the conjugate through a G-50 sephadex column with 0.1 M PO_4 pH 7.8, 10^{-4} M DTT and 10^{-3} M EDTA.

The procedure results in a conjugate containing 1-2 TNP per luciferase with a recovery of 80-90% of the original enzymatic activity.

The procedure for making TNP-glucose-6-phosphate dehydrogenase conjugates is given below:

- Mix 200 nmoles TNBS with 20 nmoles of glucose-6-phosphate dehydrogenase in 0.6 ml of 0.1 M PO_4 pH 9.0.

- Incubate 12 hours at 4°C.

- Pass the reaction through a G-50 Sephadex column and then extensively dialyze against 0.1 M PO_4 pH 7.0.

The amount of TNP bound per G6PDH was determined by optical density at 348 nm where it has a molar extinction of 15,500. There was one TNP per G6PDH molecule. The conjugate retained 80% of its catalytic activity. The conjugate is stored in 0.1 N PO_4 pH 7.8 at 4°C and has been stable for 6 months with no loss of enzymatic activity.

f.   Three Basic Approaches were examined:

   (1) Homogeneous bioluminescent immunoassay

   (2) Heterogeneous bioluminescent immunoassay

   (3) Amplified heterogeneous bioluminescent immunoassay

   1.   Homogeneous Bioluminescent Immunoassay

        This assay is schematically shown in Fig III. In this assay the antigen is covalently linked to an enzyme, i.e., firefly luciferase and, upon binding of antibody to the antigen, the enzymatic activity of the luciferase is either significantly inhibited or enhanced. Then if more free antigen (TNT) is added along with the luciferase-TNP this will compete with the luciferase-TNP for antibody binding sites and this will be measured as an increase or decrease in light intensity.

        The homogeneous bioluminescent immunoassay concept was tested using an antibody for methotrexate which was available before an antibody for TNT became available. Using this technique we prepared luciferase-methotrexate conjugates of 1:1 or 2:1 molar ratio and addition of antibody resulted in a 16% or 30% reduction in catalytic activity. This was not judged to be a significant enough inhibition to form the basis of a sensitive and reproducible assay and was not further investigated.

Figure III. Homogeneous Immunoassay

Luciferase-TNP, when in the presence of anti-TNT, forms a complex which is inhibited for light production. Addition of free TNT will displace some of the TNP-luciferase and this is measured by an increased light production in the standard assay. In practice constant amounts of luciferase-TNP were mixed with increasing amounts of free TNT. This mixture was added to antibody and the amount of light obtained was measured.

HOMOGENEOUS IMMUNOASSAY
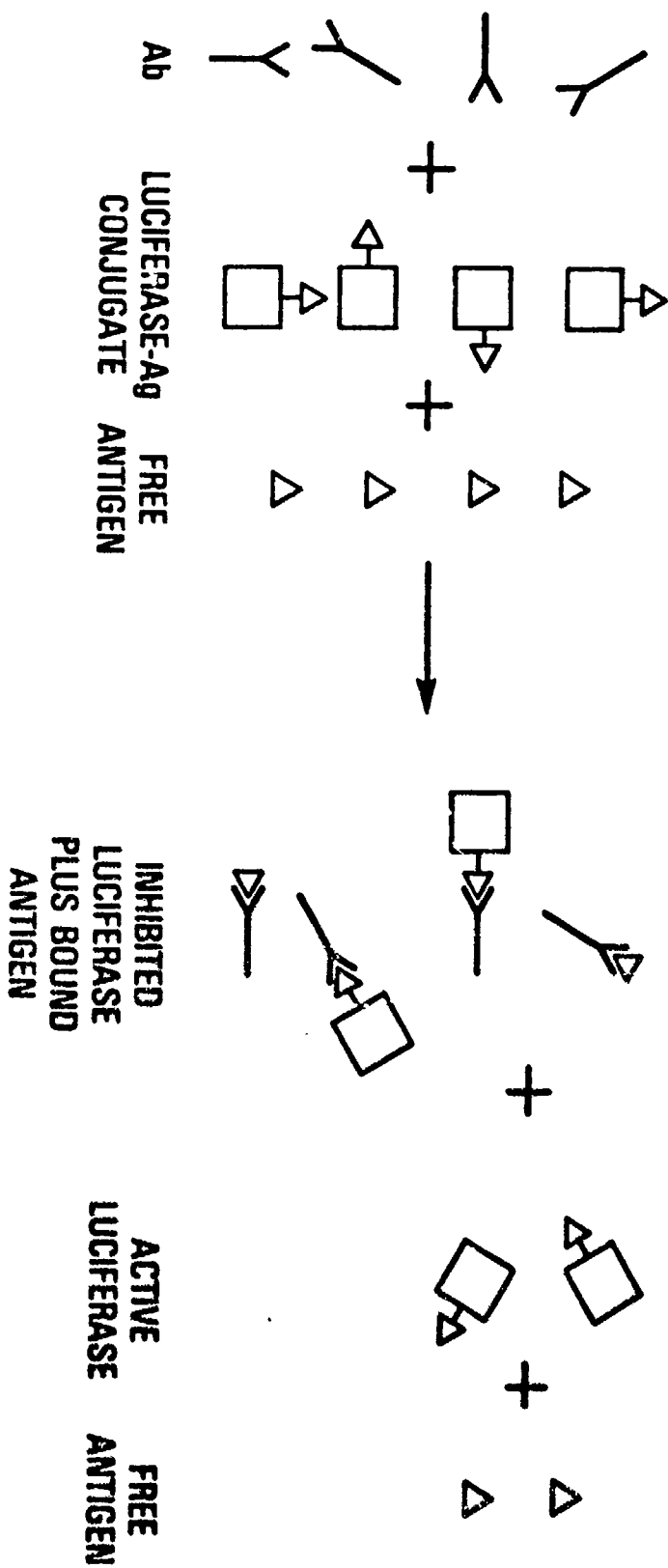
It is possible that a more careful scrutiny into conditions of binding antigen onto luciferase might still produce a viable assay and this should be further investigated. Investigation of a homogeneous immunoassay for TNT using the diaphorase enzyme is planned.

2.   Heterogeneous Bioluminescent Immunoassay

The principles of this assay are shown in Fig. IV. The essential features are: the antibody (anti-TNT, goat IgG) is covalently attached to a Sepharose matrix; the free antigen and luciferase-TNP are allowed to react with the Sepharose-antibody for a prescribed period of time; the amount of binding is a function of the concentrations of free TNT, luciferase-TNT, and the number of binding sites on the antibody, the affinities of the antibody for TNT and TNP-luciferase. After the incubation, unbound antigen and conjugate are washed away and the bound TNP-luciferase is assayed by light production. The amount of bound luciferase-TNP is inversely proportional to the amount of TNT in the sample. Using this procedure we were able to detect 50 femtomoles of free TNT (5).

3.   Amplified Heterogeneous Bioluminescent Immunoassay

The principle of this assay is shown in Fig. V. It is very similar to the procedure described for the luciferase-TNP assay except that the TNP is linked to G-6-PDH instead of luciferase and that the free TNT and the G-6-PDH-TNP are allowed to interact with the antibody in a sequential manner. In this assay we are taking advantage of the fact that G-6-PDH has a turnover number 70,000. Therefore, if we use a G-6-PDH-TNP conjugate in a competitive binding assay with TNT, it is theoretically possible to detect much lower levels of enzyme-TNP bound to the Sepharose. For example, we were able to measure 1×10^{-18} moles of G-6-PDH-TNP in solution by allowing the enzyme to react with its substrates, i.e., G-6-P and NAD(P)$^+$, for 10 minutes at 37°C. The resulting NAD(P)H produced was easily measured using the immobilized oxidoreductase and luciferase. Using this amplified procedure we were able to measure 10 attomoles of TNT (5, 6).

Figure IV. An Example of the Hetereogeneous Immunoassay

This differs from the homogeneous assay in that the antibody is immobilized
to Sepharose and the binding of TNP-luciferase to the antibody does not alter
the catalytic activity.

(1)   The immobilized antibody is allowed to incubate with TNT and TNP-luciferase
       for approximately 2 hours.

(2)   The Sepharose-antibody is then washed extensively to remove any non-
       specifically bound TNT or conjugate.

(3)   The bound luciferase is assayed by light production. The amount of
       bound TNP-luciferase is inversely proportional to the amount of TNT added.

Sepharose-Ab

TNP-Luciferase

TNT

1. Incubate
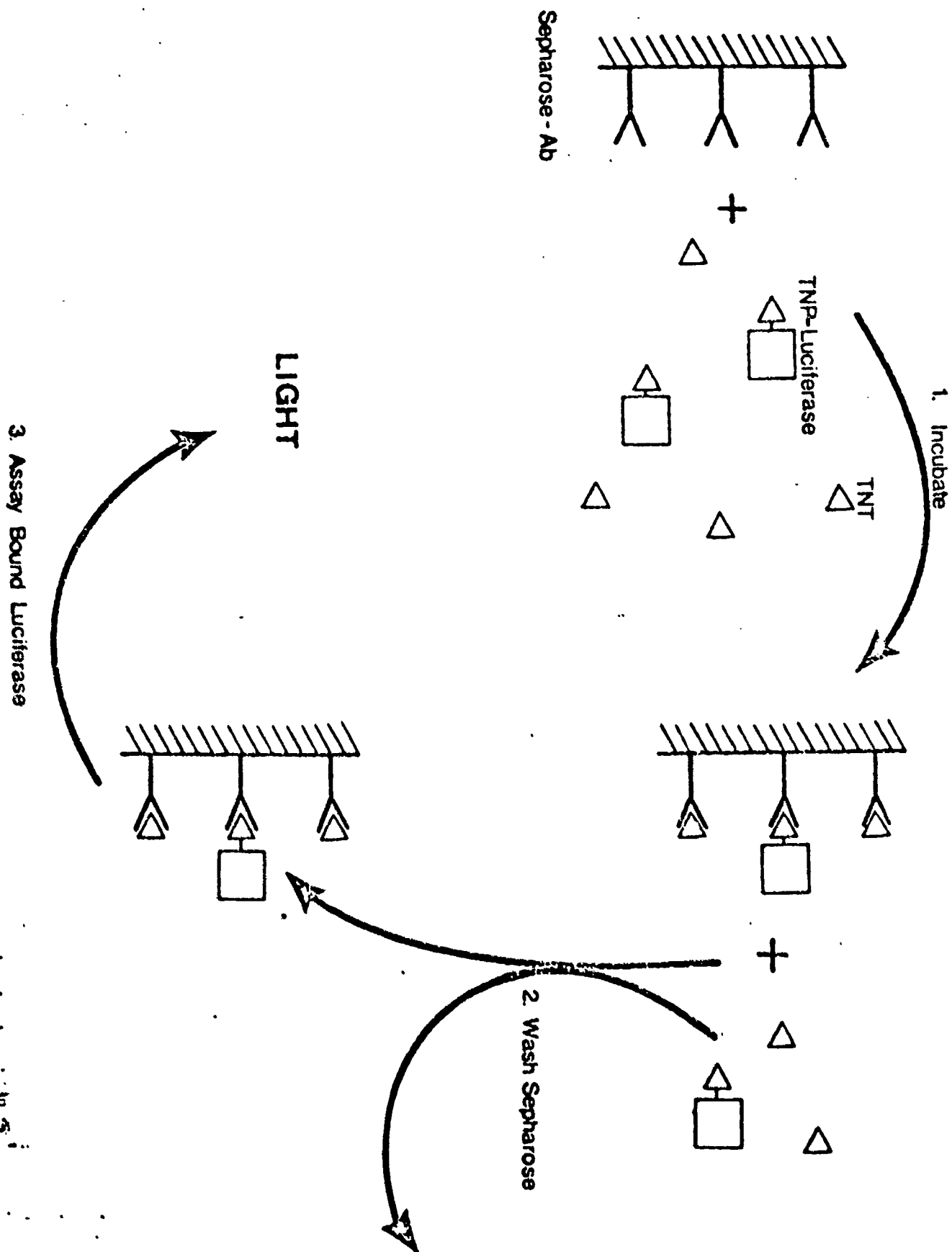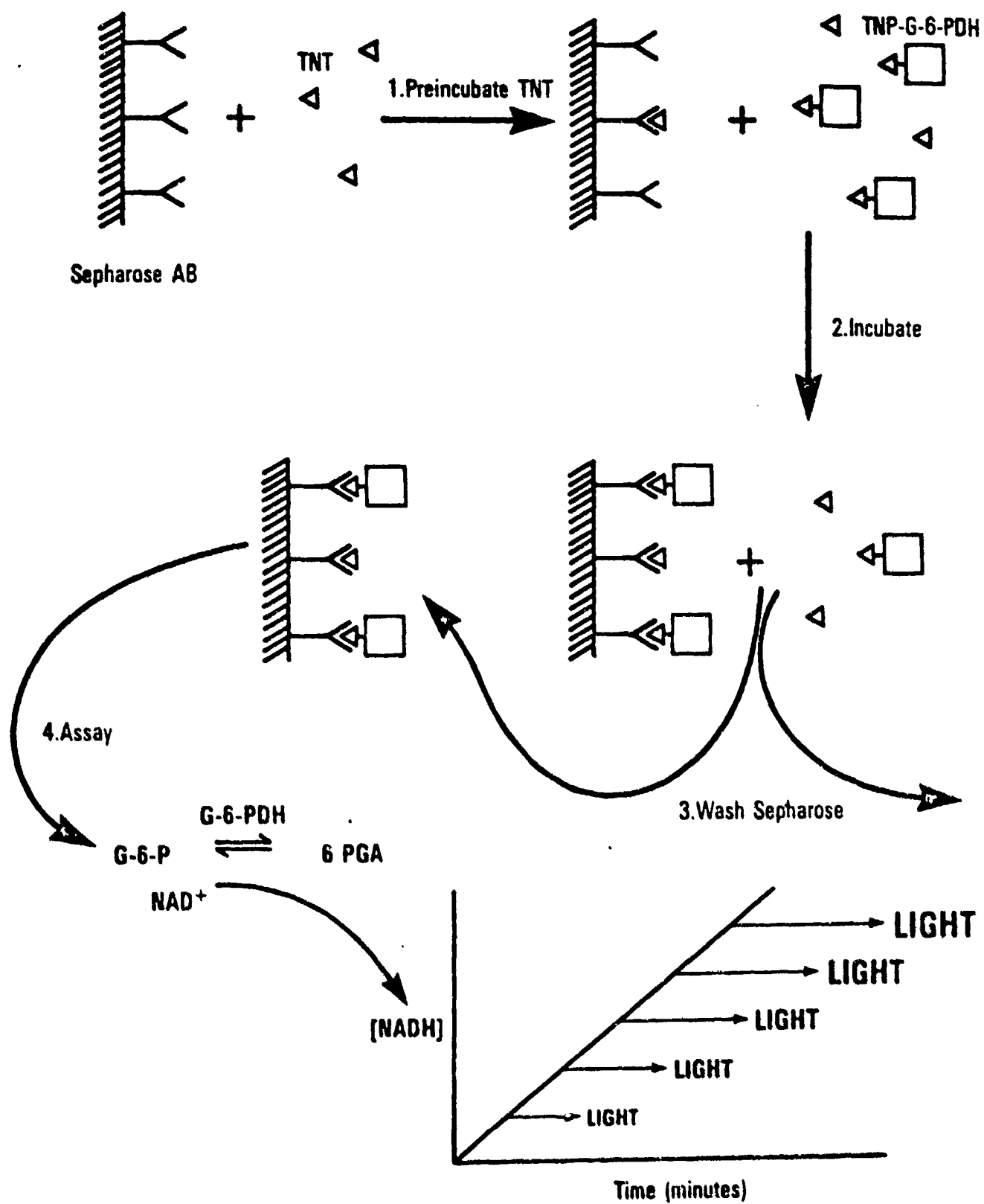
LIGHT

2. Wash Sepharose

3. Assay Bound Luciferase

Figure V. Amplified Bioluminescent Immunoassay

This assay is a 4-step procedure:

(1) The free TNT is allowed to incubate with the immobilized antibody for

30 minutes. This allows the TNT to bind preferentially at the high affinity

sites on the antibody.

(2) TNP-G-6-PDH is added and allowed to bind to any remaining free sites.

(3) The unbound or non-specifically bound TNT and TNP-G-6-PDH are removed

by extensive washing.

(4) The bound TNP-G-6-PDH is assayed by incubating 10 minutes with excess

glucose-6-phosphate and NAD.

The NADH produced is measured with the immobilized oxidoreductase and luciferase

and the light intensity is directly proportional to the concentration of NADH.

Sepharose AB

1.Preincubate TNT

TNT

TNP-G-6-PDH

2.Incubate

4.Assay

3.Wash Sepharose

G-6-P ⇌ 6 PGA

G-6-PDH

NAD⁺

[NADH]

LIGHT
LIGHT
LIGHT
LIGHT
LIGHT

Time (minutes)

g. <u>A Brief Summary of Accomplishments up to July 1981</u> is given below
(details in interim report):

1. Antibody to TNP-BSA was raised in goats and purified by ammonium sulfate fractionation.

2. The antibody was immobilized to Sepharose-4B and acid washed to remove bound antigen.

3. Immobilized antibody was characterized for total binding sites for TNT.

4. Time course of binding was established.

5. TNP-luciferase conjugates, 1:1, were prepared and found to retain 70-90% of the original enzymatic activity.

6. A heterogeneous immunoassay was developed using immobilized antibody and TNP-luciferase providing a lower detection limit of 50 femtomoles of TNT.

7. TNP-G-6-PDH conjugates (1:1) were prepared, retaining 80% of the activity of the native enzyme.

8. An amplified immunoassay using immobilized antibody and G-6-PDH-TNP was developed with a detection limit of 10 attomoles of free TNT.

9. A model system for the TNT detection was established using methotrexate as antigen. It was developed in the heterogeneous mode and permits sensitivity of about 2 pmoles.

The most significant accomplishment was the demonstration of the feasibility of detecting 10^{-17} moles (10 attomoles) of TNT. Further increases in detection sensitivity can be expected to result from optimization such as the following. Volumes of reactions can be smaller, proper instrumentation, i.e., a more sensitive phototube and different sample chamber geometry, could increase sensitivity. Longer incubations of G-6-PDH-TNP, i.e. 30-60' rather than 10 minutes, would improve detection limits. Possible use of monoclonal antibodies with high affinity constants could increase sensitivity.

While we feel the principles for a very sensitive BIA for TNT have been documented, in the present form they are not practical.

With these very low amounts of TNT and TNP-G-6-PDH conjugates, background problems are encountered. The non-specific binding of G-6-PDH-TNP to the Sepharose becomes very significant and requires extensive washing to remove such enzyme. This is time-consuming and also is subject to the possible loss of small amounts of Sepharose-Ab during the manipulations. When one is dealing with such small amounts of materials, i.e., both Sepharose-Ab and bound enzyme, this is a serious problem. At these very low levels, especially with an amplification step, the variability or non-reproducibility assumes importance not encountered with assays in the femtomole or picomole range.

Our next efforts were directed towards trying different protocols in an effort to develop a more rapid and reproducible method for measuring small amounts of TNT -- preferably in an automated system.

III. RESULTS OBTAINED FROM JULY 1981 THROUGH SEPTEMBER 1982

The research during the past 14 months was directed toward finding a method which is more rapid and reproducible in detecting small amounts of TNT. Several different approaches were examined and these are discussed in this section.

a.   Attempts to Develop a Liposomal Assay System

Recently there has been interest in the use of synthetic vesicles, liposomes, to entrap molecules which are later released by specific lysis (7). The principle is shown schematically in Fig.VI. Liposomes can be synthesized with either enzymes or substrates entrapped within the lipid layers. The liposomes are sensitized by coupling the ligand of interest (TNP) to a lipid, i.e.,

Figure VI. Principles of the Liposome Assay

(A) TNP sensitized liposomes with an enzyme (or substrate) entrapped are exposed to anti-TNP. The antibody will bind to the TNP on the liposome. When this entity is exposed to complement (B), lysis occurs and the enzyme (or substrate) is released into the medium and can be assayed. If free TNT is mixed with the liposomes initially, it will compete with the liposomal bound TNP for the antibody and the amount of lysis occurring will be decreased.
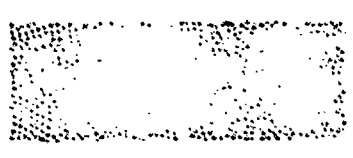
SENSITIZED LIPOSOME + ANTI-TNP

ANTIGEN (TNP)

ENTRAPPED
ENZYME

Ab

A

+
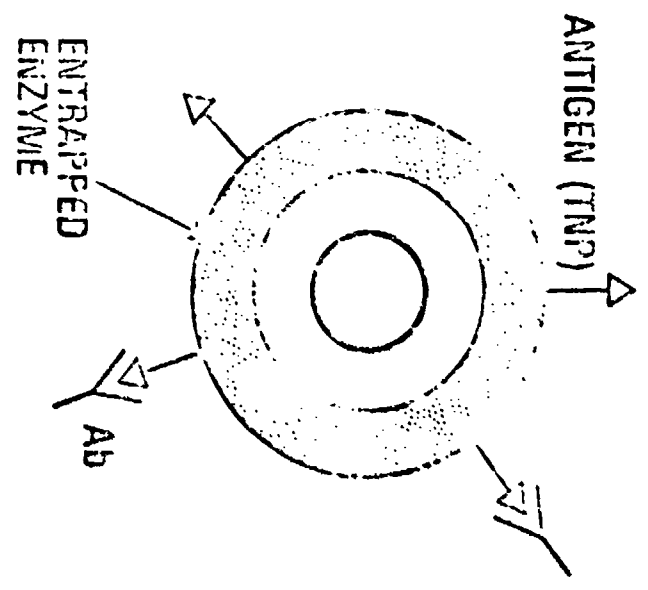
COMPLEMENT

LYSIS

ENZYME

B

phosphatidylethanolami.e. This conjugate is then added to the lipid mixture used to prepare the liposome. Upon exposure to specific antiserum, i.e. anti-TNT, and complement the liposomes are lysed releasing the entrapped marker molecule. Such a procedure has been used successfully for measuring ng levels of theophylline (7). This procedure has the advantage of not requiring a separation or washing step.

Materials and Methods

Phosphatidyl ethanolamine (bovine brain) (P.E.), phosphatidyl choline (bovine brain) (P.C.), phosphatidyl choline (egg yolk), dicetyl phosphate, cholesterol and luminol were purchased from Sigma Chemical Company, MO. Pre-coated TLC sheets (Silica gel 60 F_{254}) were obtained from MC/B Manufacturing Chemists Inc., OH. Guinea pig complement was purchased from Miles Laboratories Inc., IN.

N-Trinitrophenyl-phosphatidyl ethanolamine (TNP-PE)

Trinitrobenzenesulfonic acid (TNBS:20 mg), PE (4 ml; 10 mg/ml in chloroform) and triethylamine (15 µl) together with sufficient dry absolute methanol to dissolve the TNBS were stirred at room temperature under nitrogen for 20 h. The crude mixture was chromatographed on a silica TLC plate. The plate was developed with chloroform:methanol:water (70:30:5 v/v). TLC revealed several yellow bands. A band with R_f=0.65 was confirmed as the product by a positive iodine stain (organic substance), a weakly positive ninhydrin reaction (amine), and a positive phosphate reaction (phosphotungstate reagent). The crude reaction mixture was chromatographed on the TLC plates. The appropriate band was removed and the silica extracted with chloroform:methanol (1:1 v/v).

N-Dinitrophenyl-phosphatidyl ethanolamine (DNP-PE)

This was prepared according to the method of Uemura, X. and Kinsky, S. C. (8). The crude product was chromatographed on a

column (1.6 x 14 cm) of Bio-Sil A (Bio-Rad) and DNP-PE was eluted with $CHCL_3$:
MeOH (10:1 v/v). The purity of DNP-PE was checked by TLC on silica.

Preparation of Liposomes

The basic lipid mixture comprised phosphatidyl choline (50 mg), cholesterol
(20 mg) and dicetylphosphate (3 mg). This was dissolved in chloroform (5 ml)
containing methanol (500 μl). Sensitizer (DNP-PE or TNP-PE) was added to this
mixture in the ratio of TNP-PE or DNP-PE:PC, 1:100. This mixture was evaporated
and then resuspended in 3 ml of a Tris buffer (0.1 M, pH 8.0) containing sodium
chloride (0.05 M), magnesium chloride (1 mM) and calcium chloride (0.15 mM)
together with an appropriate concentration of marker molecules. The mixture
was then shaken vigorously and vortexed, left to stand at 4°C for several hours,
and then dialysed against the Tris buffer.

Liposomes containing the following markers were prepared: (i) firefly
luciferase (10 μl, 8.86 mg/ml); (ii) ATP (150 μl, 0.02 M); (iii) GJPDH (20 μl,
5 mg/ml); (iv) Luminol (16 mg); (v) luciferin (1 ml, 1 mM); (vi) G6P (100 mg).

Lysis of Sensitized Liposomes

The assay mix consisted of 180 μl Tris buffer (see previous Section),
100 μl liposomes, 50 μl antiserum (anti DNP or anti TNT), and 40 μl guinea pig
complement. These, together with the appropriate co-reactants, were mixed
(e.g., luciferin, magnesium chloride and ATP for firefly luciferase loaded
liposomes) and light output monitored as a function of time.

Results

Generally the results obtained with the actively sensitized liposomes
were disappointing.

Firefly Luciferase Loaded Liposomes

Treatment of the TNP liposomes with antibody/complement produced lysis
and a release of luciferase. This release was inhibited by added TNT,

however the difference was very small. The DNP-sensitized liposomes failed to show any release of luciferase when treated with antibody/complement.

ATP, G6PDH, and Luciferin Loaded Liposomes

None of these showed any lytic response to antibody/complement.

Luminol Loaded Liposomes

Some evidence was obtained for antibody/complement induced lysis. The degree of lysis did appear to be influenced by added TNT.

G6P Loaded Liposomes

Triton treatment of these liposomes demonstrated the presence of G6P inside the liposomes. DNP was shown to inhibit the antibody/complement mediated lysis of the liposomes, but the difference in signal between high and zero levels of DNP-glycine (25 μl, 20 mg/ml) was small.

Discussion and Conclusions

Generally the yield of entrapped marker was very low, and this was a major factor contributing to the poor performance of the sensitized liposomes. Other complicating factors are listed below.

i) complement or antibody on its own produced lysis of the liposomes.

ii) the high protein concentration in the lysis mixture interfered with both the luminol and firefly luciferase reactions.

iii) the anti-TNT serum may still have contained bound antigen and thus it would not have been particularly effective in initiating complement fixation. (This antiserum had not been acid washed, cf. earlier reports of the TNT immunoassay). This particular problem was by-passed by using a commercial anti-DNP serum and DNP-sensitized liposomes. Results obtained with these liposomes were, however, no better.

Other workers have been successful with liposomes in immunoassay. However, the instability of liposomes, the large number of reagents and the lengthy incubations required suggest that, even if perfected, immunoassays using sensitized-liposomes would be unsuited to routine use either in the laboratory or in the field.

b.   Studies with Two-Phase Systems in the Separation of Antibody-Bound and Free TNT

We investigated the use of a two phase system in hopes of developing a faster and more simplified immunoassay. Certain aqueous polymer solutions, when mixed together, produce a turbid solution which subsequently separates into two distinct phases. Many examples of two-phase systems have been described involving polyethylene glycol (PEG). PEG/dextran as well as mixtures of polymers and salts, e.g., PEG/potassium phosphate (9).

The differing composition and therefore differing properties of various two-phase systems has been widely used in the separation and partitioning of molecules between the two phases. The partitioning of a molecule is determined by the properties of the individual phases and the molecule itself, i.e. charge, hydrophobicity. The properties of the phases can be manipulated in a number of ways. Addition of salts will effect the partitioning of charged molecules. Alternatively the properties of one of the phase components may be selectively modified. Application of a two-phase system as a separation procedure in immunoassay was first described by Mattiasson (10). He reported a partition affinity ligand assay (PALA) for digoxin. He used a 30% W/W PEG: 30% W/W $MgSO_4$ (1:3 v/v). Free digoxin partitioned preferentially into the upper, more hydrophobic PEG phase, but the complex of digoxin with anti-digoxin partitioned into the $MgSO_4$ lower phase. This difference in the partitioning of the free and bound ligand was of a sufficient magnitude to allow its use in an assay for digoxin. Since this is a rapid nonequilibrium assay, the amount of time of contact between antibody and antigen must be standardized.

Experiments and Results Obtained with TNT and DNP

The general equation is shown below:

$$TNT + TNT* + Ab \rightleftharpoons TNT \cdot Ab + TNT* \cdot Ab$$
$$\text{free} \qquad\qquad\qquad \text{bound}$$
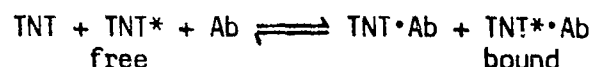
*TNT is labelled with either luciferase or G-6-PDH.

We investigated the following two phase systems.

PEG 4000/MgSO$_4$

PEG 8000/MgSO$_4$

PEG 20,000/MgSO$_4$

PEG 4000/Dextran

PEG 20,000/Dextran

PEG 20,000/Dextran sulfate

Dextran Sulfate/PVP

Dextran Sulfate/Dextran

Dextran Sulfate/DEAE Dextran

Effect of the Phase Components on the Activity of Firefly Luciferase and G-6-PDH

Since these enzymes were to be used as labels for TNT the effects of the various two-phase systems on the catalytic activity was investigated. Table I shows the data obtained for G-6-PDH. Only dextran exhibited any significant inhibition. Table II shows the effects on firefly luciferase. In MgSO$_4$ the enzyme was significantly inhibited; this was due to the known inhibitory effect of sulfate anions on the enzyme. PEG solutions stimulate enzyme activity and dextran showed some inhibition.

Partition Experiments

Either DNP-G6PDH or DNP-luciferase conjugates were used to study the partion of labelled antigen between different phases. An aliquot of labelled antigen was added to the two-phase system. The mixture was vortexed for one

Table I. Effect of Different Components of Phase Systems on G6PDH Activity

COMPONENTS	%
Control (water)	100
12.5% w/w magnesium sulfate	112
30% w/w magnesium sulfate	85
12.5% w/w PEG 8000	92
30% w/w PEG 8000	86
12.5% w/w PEG 20,000	130
30% w/w PEG 20,000	120
15% w/w dextran sulfate 173,000	60

Table II. Effect of Different Components of Phase Systems on Firefly Luciferase Activity

COMPONENTS	%
Control (0.1 M phosphate pH 7.0)	100
12.5% w/w magnesium sulfate	115
30% w/w magnesium sulfate	24
12.5% w/w PEG 8000	82
30% w/w PEG 8000	270
12.5% w/w PEG 20,000	196
30% w/w PEG 20,000	130
15% w/w dextran sulfate 173,000	48

minute and then centrifuged in order to accelerate phase separation. Enzyme activities were then measured in the upper and lower phases. Some typical results are shown in Table III and IV where the relative volumes of the phases were varied as well as the pH.

Table V shows that the effects of adding various salts resulted in only small changes in the partitioning of TNP-G-6-PDH.

Table VI demonstrates that increasing antibody concentration does increase the ratio of K/K_{Ab} in one case to 41. This should be pursued more carefully to determine if it is possible to further alter the distribution of the conjugate in the presence of antibody.

c.   Non-Equilibrium Bioluminescent Immunoassays

Introduction

Mattiasson and co-workers have described an automated non-equilibrium immunoassay of gentamicin which uses small columns packed with immobilized anti-gentamicin in a flow system. An enzyme - gentamicin conjugate (catalase-gentamicin) is used and is detected by the heat generated when it acts upon a substrate (peroxide).

Based on this work an automated flow system for TNT and methotrexate immunoassays was constructed which used small flow cells packed with either Sepharose immobilized anti-TNT or anti-methotrexate. A flow cell packed with Sepharose - co-immobilized bacterial luciferase and NADH:FMN oxidoreductase was used to detect NADH generated by TNT- or methotrexate-G6PDH conjugates. The design of the flow system is shown in Figure VII.

The immobilized antibodies are exposed to a mixture of antigen and antigen-G6PDH conjugate. Bound conjugate is detected by exposing the bound conjugate to G6P and NAD. The NADH formed in the enzyme reaction is then detected

Table III. Partition of DNP-G6PDH in a Dextran Sulfate:DEAE Dextran System

pH	No Antibody	Added Antibody
	Partition Coefficient K*	Partition Coefficient K
6	228	40
7	108	44
8	165	39

*K = $\dfrac{\text{concentration of conjugate in the upper phase}}{\text{concentration of conjugate in the lower phase}}$

Table IV: Partitioning of DNP-G6PDH in a PEG: Magnesium Sulfate System

Two-Phase System	No Antibody Partition Coefficient K*	Added Antibody Partition Coefficient K
PEG 8000 (30% w/w):MgSO$_4$ (30% w/w)		
1:1 v/v	0.04	0.15
1:3 v/v	0.5	0.12
3:1 v/v	0.005	0.004

*K = concentration of conjugate in the upper phase
     concentration of conjugate in the lower phase

Table V. Effect of Salt on the Partition of TNP-G-6-PDH
Partition in a PEG:MgSO$_4$ System

Additions:*	No Ab	Added Ab	K/KAb
+ 500 µl H$_2$O	K = 0.15	K = 0.03	5
+ 500 µl 2 m NaCl	K = 0.01	K = 0.007	1.4
+ 500 µl 2 m KCl	K = 0.007	K = 0.01	0.7
+ 500 µl 0.5 m Na phosphate pH 7.0	K = 0.12	K = 0.04	3

*All systems were 1:3 v/v of a 30% PEG 8K/30% Mg SO$_4$ (w/w).

Table VI. <u>Effect of Increasing Antibody Concentration on the Partition</u>

of TNP-G-6-PDH in PEG/MgSO$_4$ System*
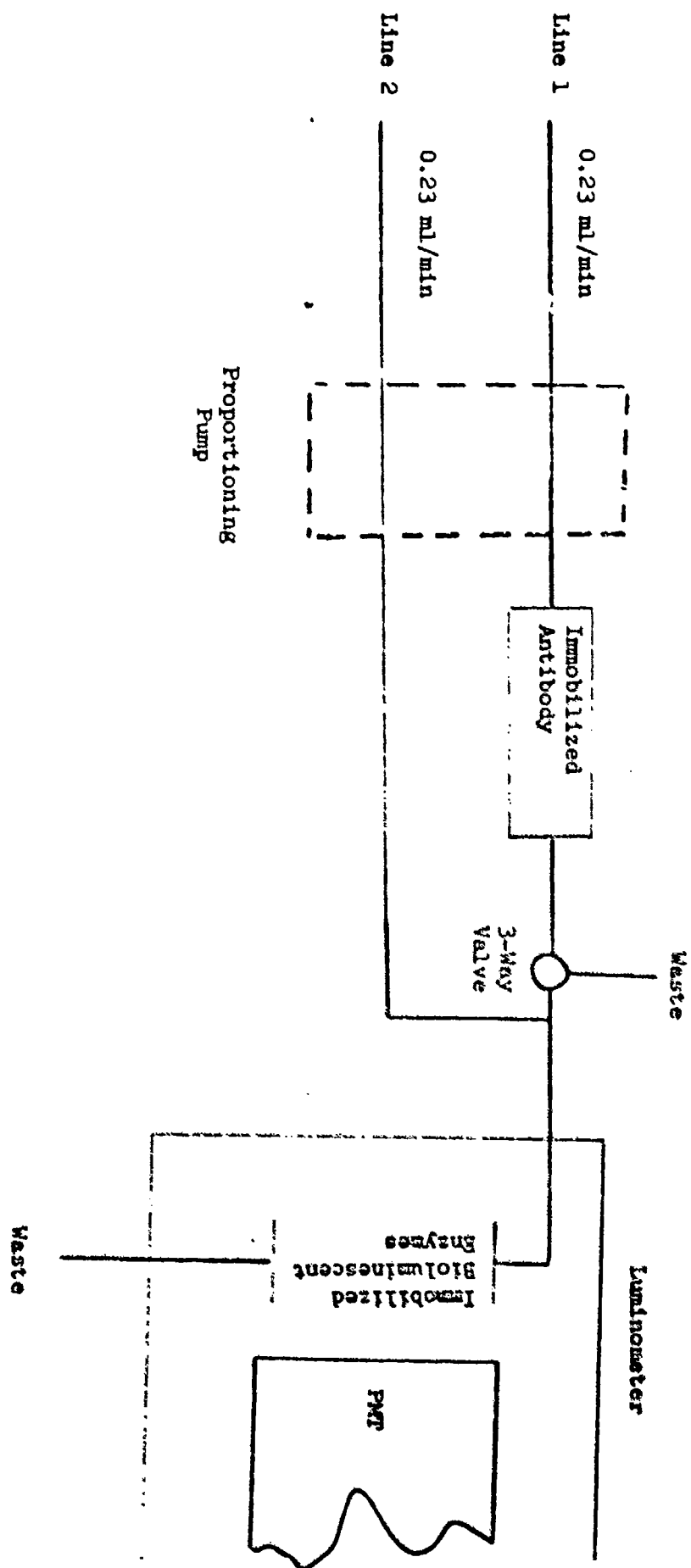
	<u>Ab = 0</u>	<u>Ab = 10 µl</u>	<u>Ab = 20 µl</u>	<u>K/KA</u>
PEG 8K	0.7	0.13	0.017	41.2
PEG 20K	2.04	0.33	0.22	9.2

*All systems were 1:3 v/v of a 30% PEG/30% MgSo$_4$ (w/w). Final volume was 4 ml.

<u>Figure VII.</u>   Schematic of the Automated Flow System Used for the
Non-Equilibrium Immunoassays.

For details of the operating sequence see Table VII.

FIGURE VII. Design of the Automated Non-Equilibrium Bioluminescent Assay Flow System

by light produced with the co-immobilized bacterial luciferase/oxidoreductase which is located in front of a photomultiplier tube inside a luminometer.

The timing sequence and reagents used in the assay are shown in Table VII. KCl is used to remove any substances non-specifically bound to the immobilized antibody. The substrate solution is divided into two parts in order to minimize the number of reagents which pass through the flow cell containing the immobilized antibody. A three-way valve is included downstream from this flow cell in order to minimize the number of reagents which pass through the flow cell containing the co-immobilized bioluminescent enzymes. The immobilized antibody flow cell is regenerated between samples using glycine-HCl (0.2 mole/l, pH 2.2). This is followed by 1 M phosphate buffer (pH 7.0) in order to ensure that any acid remaining in the flow cell is neutralized.

Materials and Methods

Immobilized Antibodies. The IgG fraction of anti-methotrexate or anti-TNT was immobilized onto cyanogen bromide activated Sepharose 4B as indicated in an earlier section and described in the interim report of July 1981 as well as in the open literature (5, 6).

Co-Immobilized Bacterial Luciferase and NADH:FMN Oxidoreductase. This was prepared as described by Ford and DeLuca (11).

TNP-G6PDH and Methotrexate G6PDH Conjugates. These were prepared as described above and in the interim report of July 1981 and in the open literature (5, 6). The methotrexate-G6PDH conjugate contained 125 µg G6PDH/ml.

Construction of Flow Cells. The body of the flow cells was a 2 cm long piece of glass tubing. It was filled with 100 µl of the Sepharose-immobilized antibody or the enzymes respectively which were constrained within the cell by two plastic frits. A piece of Tygon tubing was then fitted into each end of the flow cell. If necessary the joints of the flow cells were sealed with glue.

Table VII. Sequence of Reagents Used in the Flow System

	Line 1 (B)	Line 2
(1)	Sample, e.g., 0-100 µl methotrexate (4 mg/10 ml and 100 µl of a 125-fold dilution of methotrexate-G6PDH in 900 µl TES buffer, 30 secs	0.05 M TES wash buffer (pH 7.5)
(2)	TES wash buffer, 1 min	"
(3)	1 M KCl, 1 min	"
(4)	TES wash buffer, 1 min (A)	"
(5)	Substrate I, 40 secs	Substrate II, 40 secs
(6)	TES wash buffer, 3 min (B)	TES wash buffer
(7)	0.2 M Glycine-HCl (pH 2.2), 1 min	"
(8)	1 M Phosphate buffer (pH 2.2), 1 min	"
(9)	TES wash buffer, 1 min	"

3-way valve set so that effluent from antibody flow cell (A) passes and (B) does not pass through the immobilized enzyme flow cell.

Reagents. (See Table VII for additional details.)

Substrate Solution I. G6P (0.5 ml, 0.2 M) and NAD (0.5 ml, 20 mM) in 5 ml TES buffer (0.05 M, pH 7.5).

Substrate Solution II. FMN (200 μl, 10^{-3} M) and stock decanal emulsion (100 μl) in 5 ml TES buffer (0.05 M, pH 7.5).

Flow System. A Technicon proportioning pump was used to pump the various reagents through the flow cells. The manifold and pump rates are shown in Figure VII .

Results

The analytical response of the flow system (light-time curves) was excellent and some typical traces are shown in Fig. VIII.

TNT Immunoassay

Initially the immobilized antibody and the co-immobilized bacterial luciferase/oxidoreductase were mixed together in a single flow cell. The acid wash however killed the enzyme activity and subsequently separate flow cells for the antibody and enzyme were used.

A major problem encountered with the anti-TNT flow cell was a fall in apparent binding capacity after repeated exposure to conjugate (e.g., Fig. IX). Independent testing of the enzyme flow cell using an NADH solution revealed that its response was constant thus confirming that the problem lay in the antibody flow cell. The most probable cause was thought to be saturation of the immobilized antibody by antigen. Different antigen:antibody disrupting agents were investigated, i.e., 6 M urea, 1 M acetic acid, 1 M acetic acid containing 10% ethylene glycol, 1 M ammonium hydroxide, 1 M ammonium hydroxide containing 10% dioxane, and 0.05 diethylamine (pH 11.8). None of these was effective.

Figure VIII. Data Obtained Using the Automated, Non-Equilibrium, Flow
Immunoassay System

(A) Light obtained from a sample of 29 femtomoles of TNP-G-6-PDH.

(B) 0.1 pmole of Methotrexate - G6PDH and 1) 88 pmoles, 2) 44 pmoles and
     3) 22 pmoles of free Methotrexate (MTX) were mixed and allowed to flow
     through the automated system. Increasing the amount of free Methotrexate
     decreases the amount of MTX-G6PDH bound and the light obtained is thus
     reduced.

FIGURE VIII Analytical Traces Obtained Using the Automated Non-Equilibrium Bioluminescent Immunoassay Flow System.

A. 29.5 femtomoles TNP-G6PDH

B.

Relative
Light Intensity
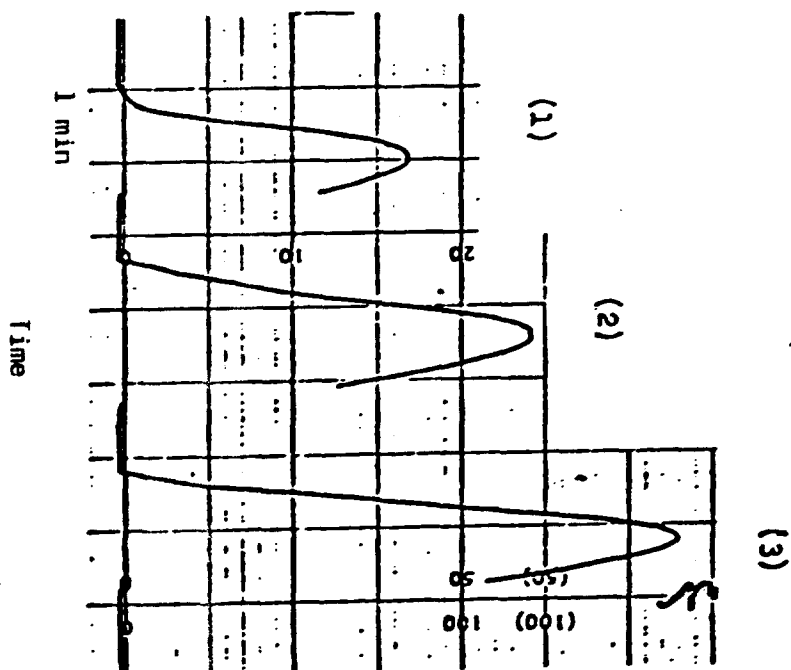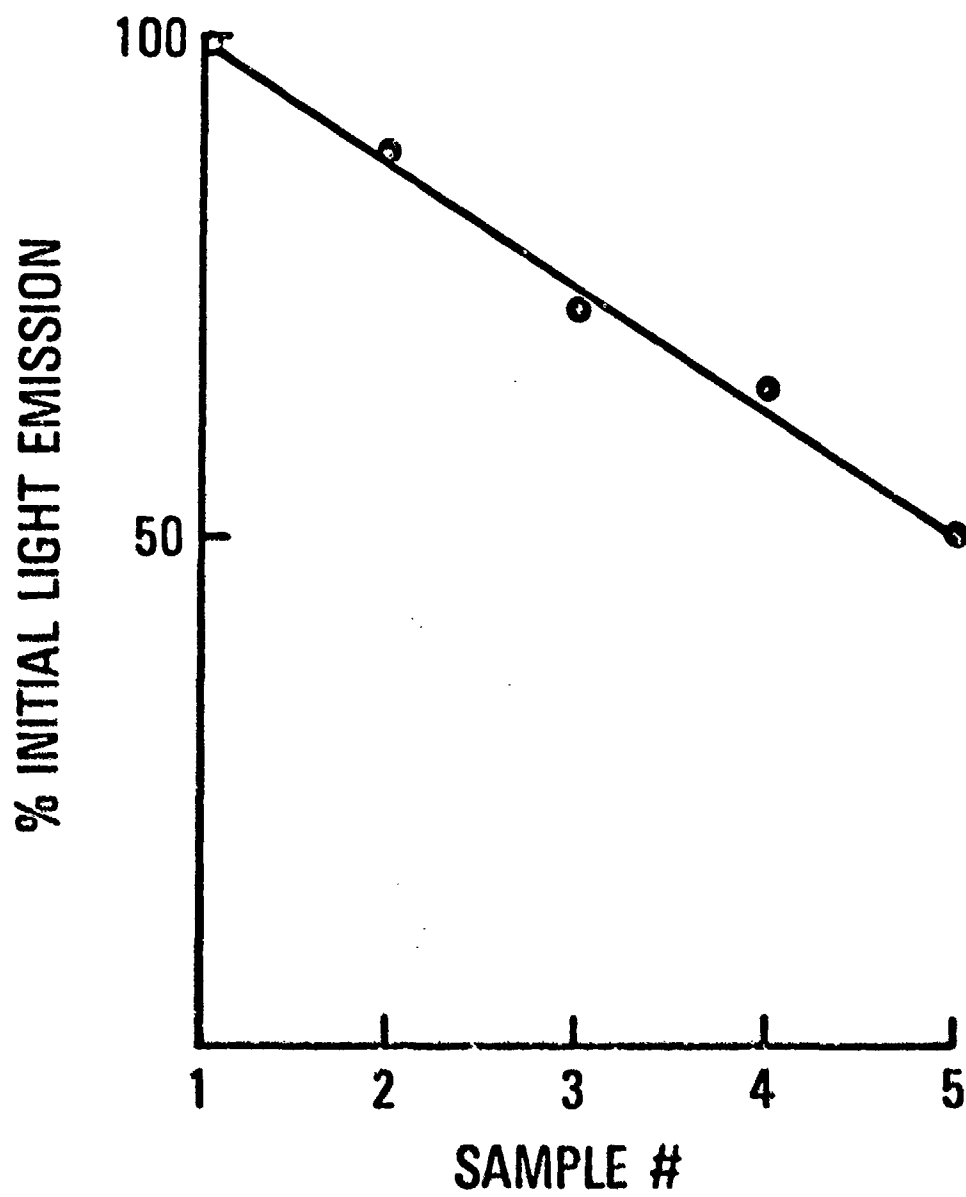
1 min

Time

(1)

(2)

(3)

1 min

Time
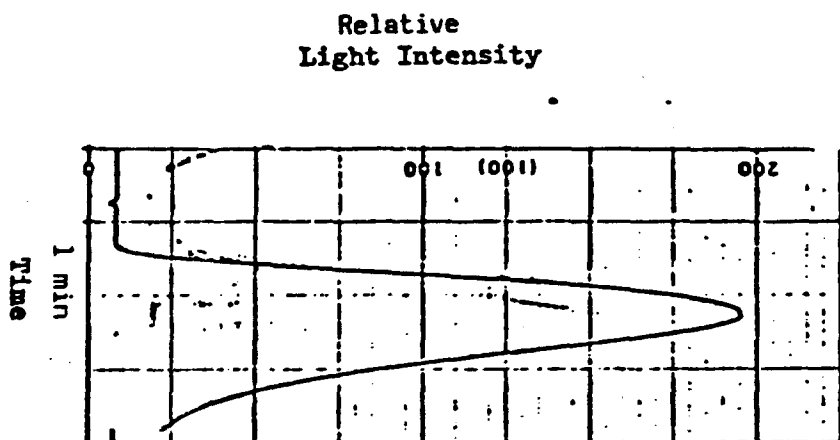
Figure IX. Light Intensity Obtained with the Automated, Non-Equilibrium, Flow
System with Repetitive Samples of 29 Femtomoles of TNP-G-6-PDH.

The amount of bound active TNP-G-6-PDH is decreasing with repetitive samples.

Previous work had shown that disruption of TNT:anti-TNT complexes was difficult to achieve. An alternative antigen was thus selected for further study of the non-equilibrium bioluminescent immunoassay.

Methotrexate Immunoassay

The anti-methotrexate flow cell did not exhibit a fall in apparent binding capacity after repeated exposure to conjugate. A competitive response was obtained in the presence of methotrexate (Fig. X). The dose-response was however over a very narrow range of methotrexate concentration. Reduction in the concentration of conjugate did not improve matters.

Conclusion

This limited study demonstrated the feasibility of an automated non-equilibrium bioluminescent immunoassay. For analysis purposes a disadvantage is the narrow range of concentration over which a dose-response relationship is obtained. For detection purposes, however, this is not of major concern. These non-equilibrium bioluminescent immunoassays required about 10-12 minutes. Further reduction of the assay times is of course desirable. More work is needed to make this approach a viable technique. For further progress in TNT detection with this technique the apparent loss of binding capacity of the immobilized antibody when exposed repeatedly to TNT must be remedied or throw away one time use of flow cells must be considered.

In the course of this study we have been able to reproducibly measure NADH in the automated flow system (Fig. XI). This means, if we can successfully develop the immunoassay with TNP-G-6-PDH, the resulting NADH produced can be conveniently measured with automation.

Figure X. Automated Non-Equilibrium Immunoassay of Methotrexate

Competitive binding of methotrexate and methotrexate-glucose-6-phosphate

dehydrogenase. The details of the procedure are given in Table VII.

AUTOMATED NON-EQUILIBRIUM
IMMUNOASSAY OF METHOTREXATE
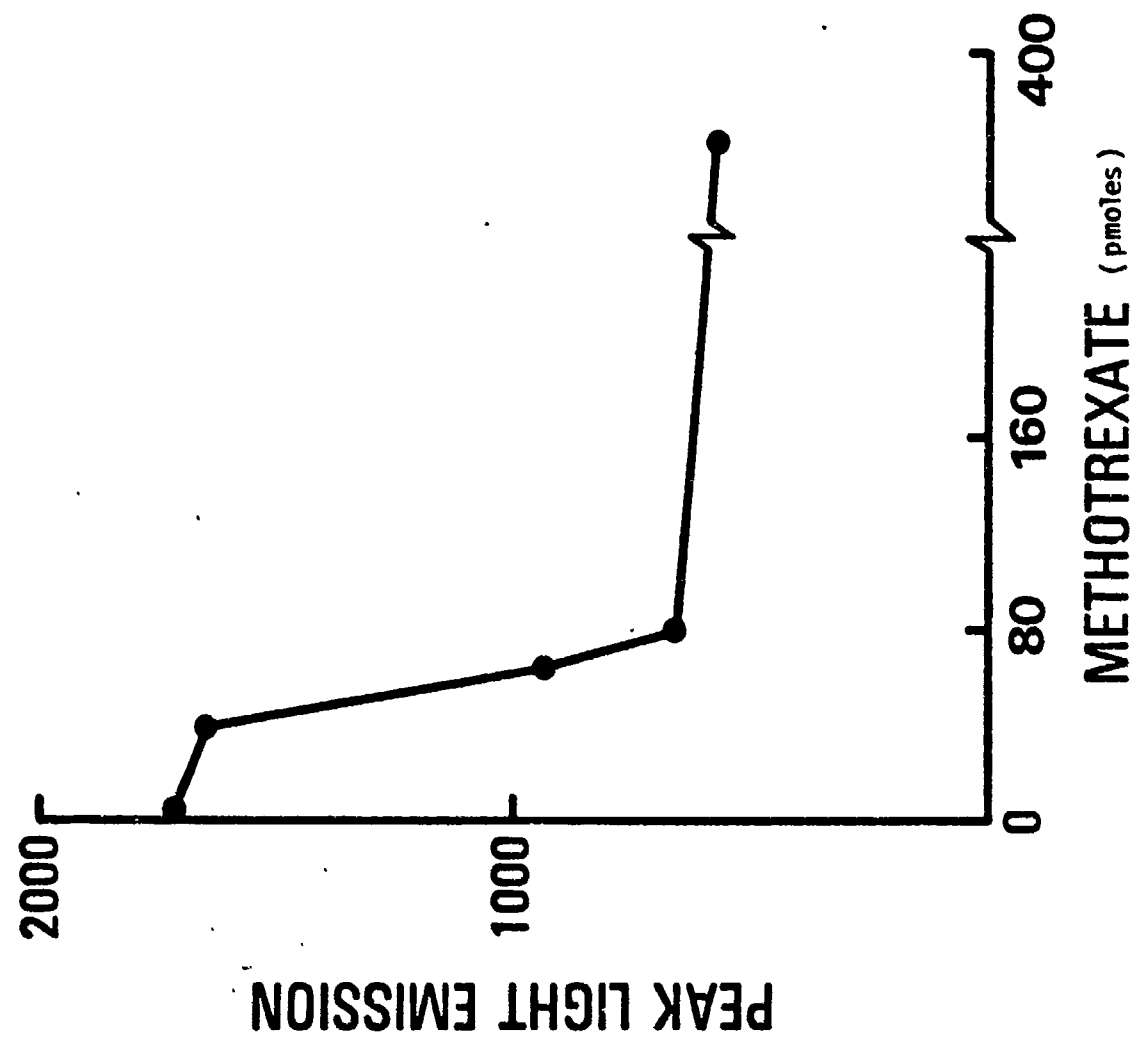
PEAK LIGHT EMISSION

METHOTREXATE (pmoles)

Figure XI. Light Obtained from Repeated Analysis of 300 pmoles of NADH Using

the Immobilized Oxidoreductase-Luciferase in the Automated Flow System.

(A) 20 second wash between samples.

(B) 80 second wash between samples.

The assay mixture contained 100 µl of a decanal-water emulsion, 200 µl of

1.5×10^{-4} M FMN in 10 ml TES buffer, 0.05 M, pH 7.5.
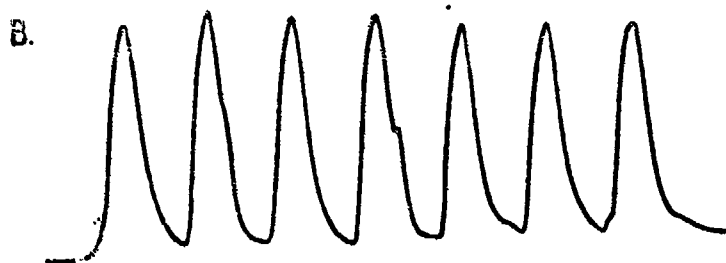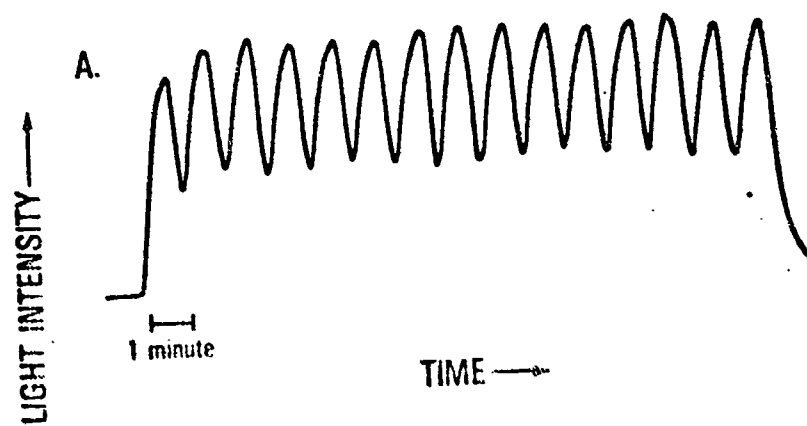
d.   Diaphorase Studies

One of the problems associated with the use of immobilized oxidoreductase and luciferase for detecting NADH is the fact that one obtains very small amounts of oxidoreductase from the luminescent bacteria. Usually only 2-3 mgs of enzyme are obtained from 1 lb of bacteria. This means that the purification and preparation of the immobilized enzymes is very time-consuming. Therefore we decided to find out if commercially available diaphorases would function in the coupled assay with the luciferase.

A Boehringer diaphorase was found to have some activity as an NADH:FMN oxidoreductase.   The enzyme was assayed for contaminating dehydrogenase activity and no activity was found for lactate, aldehyde, alcohol or alanine. A trace of malate dehydrogenase was found but this should not interfere with assays of G-6-PDH.

The Boehringer diaphorase was co-immobilized onto Sepharose 4B along with luciferase using the techniques described in the interim report July 1981, and this preparation was compared to a preparation of immobilized NADH-FMN oxidoreductase-luciferase. Both preparations gave essentially identical light output in response to NADH. The linear range was from 0.1 pmole NADH to 1000 pmoles. The major difference is that the diaphorase can oxidize DTT while the oxidoreductase cannot. Since our immobilized enzymes are usually stored in DTT, another reducing agent had to be found. Either β mercaptoethanol or reduced glutathione could be used in place of DTT as these are not active as substrates.

Conclusion: In the future we plan to use the immobilized diaphorase-luciferase for measuring NADH as this system is much more readily available.

e. Development of Microtiter Plate Immunoassay

The bioluminescent immunoassay for TNT using antibodies immobilized to Sepharose has major drawbacks in that it is time-consuming and tedious. In this form, the assay cannot be easily automated. We are developing a procedure which modifies this technique and doe. allow for subsequent automation. Many immunoassays currently in clinical use employ antibodies immobilized on polystyrene in the form of tubes or multiple well plates. We chose microtiter plates due to their uniformity and ease of manipulation of large numbers of samples.

Essentially, the antibody to TNT is bound to the wells of the plate in an active form according to published procedures (12). Unbound antibody is washed out with buffer. Free TNT is added to the wells and allowed to incubate followed by incubation with TNP-G6PDH. After further incubation, all unbound TNT and TNP-G6PDH are washed out and a buffer solution containing G6P and NAD is added to each well and incubated at room temperature for a predetermined length of time. Samples are then removed from the wells and assayed (for NADH) using the immobilized oxidoreductase and luciferase. When standard curves are run using variable amounts of TNT and constant TNP-G6PDH, the light produced is inversely proportional to the amount of TNT present. See Fig. V (Schematic).

Materials and Methods

Immobilization of Antibodies on Microtiter Plates

Purifed antibody is stored frozen in aliquots in dialysis buffer: 2 M KCl, 0.1 M K_2HPO_4, pH 7.0. Protein concentration is typically 50-100 mg/ml. To coat the wells, an aliquot is thawed and diluted (1/1000-1/10,000) in freshly prepared 0.05 M carbonate/bicarbonate buffer (pH 9.5). 0.1 ml of this solution is added to each well and the plates are incubated at 4°C overnight.

After incubation, the unbound antibody is washed 3x with Phosphate Buffered Saline containing 0.5 ml/l Tween 20. Plates are stored in PBS plus NaN_3 at 4°C.

Optimized Bound G6PDH Assay

Microtiter plates are prepared as described using 10 to 100 ng anti-TNT IgG per well. TNP-G6PDH conjugate stock (typically 2-2.5 nmol/ml) is diluted appropriately in 0.05 M KPO_4, pH 7.0 containing 2 mg/ml Human Serum Albumin. 0.1 ml of this solution is carefully placed in the bottom of each well. For competitive assays with free TNT, samples are incubated in the wells for an appropriate amount of time (e.g., 30 min) before the addition of TNP-G6PDH. The plates are incubated in an incubator-shaker at room temperature for 2 hrs. After incubation, the plate is inverted and shaken to remove fluid and a strictly-timed washing protocol is begun immediately. The wells of the plate are filled with cold 0.01 M K_2HPO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.4. The plate is then allowed to soak for 1 min and then emptied. This fill/soak is timed to 1.5 min and repeated 10x for a total of 15 minutes. After the last wash the plate is inverted on clean tissue paper and patted firmly to remove any droplets of wash buffer. Immediately, the plate is righted and 300 µl substrate solution is added to each well individually at 15 second intervals. Substrate solution is $4x10^{-4}$ M glucose-6-phosphate, $2x10^{-4}$ M NAD in 0.05 M TES Buffer, pH 7.5. (TES: N-tris (Hydroxmethyl) methyl-2-aminoethanesulfonic acid). After exactly 10 min (incubate at room temperature) the solution in the well is mixed by filling and emptying 10x a 100 µl automatic pipette, then 100 µl is removed to a 6x50 mm disposable test tube (cuvette). This operation requires about 12 sec per well, hence the 15 sec intervals on filling the wells. It is essential that the pipette tip does not touch the interior surface of the

well since this can dislodge bound enzyme into the sample which would continue to produce NADH. A 10 min incubation time allows the assay of up to 40 samples. Once the solution is removed from the well, its NADH concentration remains stable for at least 30 min allowing all of the samples to be assayed for NADH after the entire 40 wells have been incubated with substrate solution.

Assay of NADH with Luminescent Immobilized Enzymes

The following sequence of additions to and manipulation of samples has been adopted in order to maximize reproducibility of light output at very low NADH levels.

- Sample (100 µl) in 6x50 mm disposable tube
- Add 10 µl 1.5×10^{-4} M FMN in distilled H_2O
- Add 300 µl TES-decanal solution, pH 7.5 (prepared by adding 100 µl of a fresh suspension of 5 µl decanal in 10 ml distilled H_2O to 10 ml 0.05 M TES Buffer, pH 7.5)
- Mix and add 50 µl of suspension of immobilized enzymes to cuvette
- Immediately invert cuvette 2x to mix and insert into LKB 1250 luminometer
- Record light output for approximately 30 seconds on chart recorder

Repeat above with all samples. Several 100 µl samples of substrate solution are also assayed for "background" light output. Substrate solution is prepared fresh daily from concentrated stock solutions of G6P and NAD which are stored frozen. Decanal suspension in distilled H_2O is also prepared fresh daily and kept on ice.

Results

Binding Capacity of Microtiter Plate Wells for Anti-TNT

In order to determine the IgG binding capacity of the wells, serial dilutions of anti-TNT IgG (goat) were made in 0.05 M carbonate/bicarbonate buffer, pH 9.5 and 0.1 ml placed in each well. The plate was incubated overnight at 4°C and all unbound antibody was washed out with phosphate buffered saline, pH 7.4. To detect goat antibodies, a constant amount of rabbit-anti-goat antibody conjugated to horseradish peroxidase was added to each well and incubated for two hours at room temperature. All excess rabbit-anti-goat-horseradish peroxidase was washed out with PBS. The amount of conjugate remaining in the wells, which is proportional to the amount of goat antibody in the wells, is measured by adding hydrogen peroxide 2.5 mM and the chromogen ABTS [2,2'-azino-di(3-ethyl-benzthiazolin-6-sulfonate)] 3.0 mM in acetate, phosphate buffer, pH 4.5. After 30 minutes, the resultant green color was measured at 415 nm. Wells containing no goat IgG were run as controls. Fig. XII shows that the wells saturate at a level of 10 µg goat IgG added.

### TNP-G6PDH on Control Microtiter Plates

TNP-G6PDH conjugates bind to blank microtiter plates which do not contain anti-TNT. This non-specific binding is slowly reversible and is proportional to the concentration added. Initially we tried to overcome this problem with "blocking" or "quenching" agents such as had been reported in the literature. Several proteinaceous blockers were tried (Bovine Serum Albumin, Human Serum Albumin, Ovalbumin, Gelatin and non-specific Immunoglobulins) as well as small molecules of hydrophobic character (Tyrosine, Phenol, Polethylene Glycol 600). None of these compounds significantly reduced the non-specific binding of the TNP-G6PDH.

Having noted that longer periods of washing or soaking of the plates resulted in less variability between wells when bound conjugate was assayed,

Figure XII.   Binding Capacity of Microtiter Plate Wells for Goat IgG

Anti-TNT goat IgG immobilized as in text. After removal of unbound IgG by
washing (PBS), plate wells were incubated with 0.1 ml 1:1000 dilution of
39 U/ml rabbit anti-goat horseradish peroxidase, 2 hrs at 25°C. Unbound
conjugate was washed out with PBS and 250 µl substrate solution* added.
After 30 min, well contents were mixed and 200 µl removed and diluted to
1.0 ml with PBS. The absorbance was measured at 415 nm.

*3.0 mM ABTS, 2.5 mM H_2O_2, 0.1 M sodium acetate, 0.05 M NaH_2PO_4, pH 4.5.
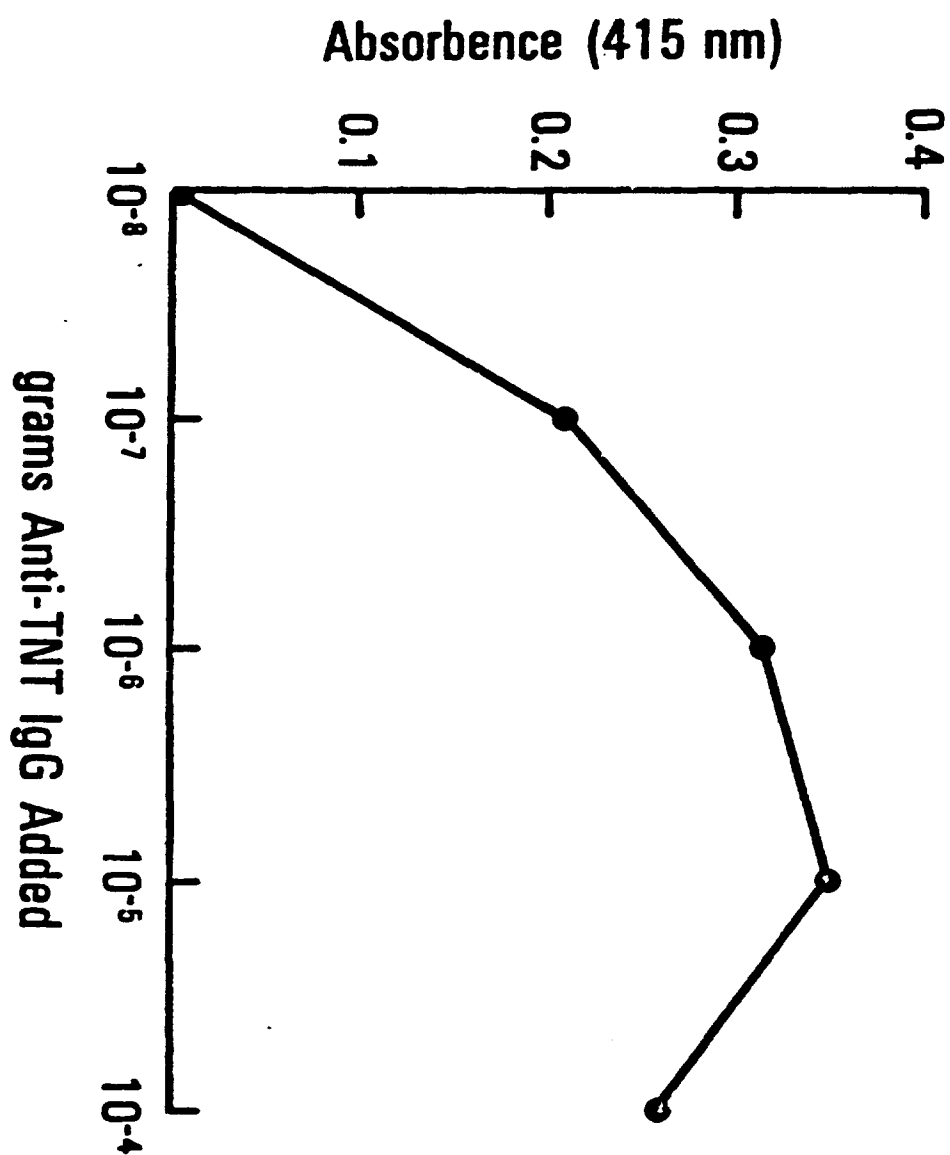
a strict washing protocol was adopted which reduced the background (non-specifically bound G6PDH) to an acceptable level and also kept a manageable time frame. The protocol requires 10x1.5 minute washes of the plate in cold PBS plus Tween. "Blank" wells containing no anti-TNT IgG are included at each conjugate concentration in order that this background can be substracted from total light to give light produced by specific TNP-G6PDH/anti-TNT interactions.

Binding Capacity for TNP-G6PDH of Anti-TNT Microtiter Plates

In order to determine the proper amount of IgG needed in the wells, a qualitative competitive assay with free TNT was run. A plate was prepared with 10 μg αTNT IgG per well. TNT concentration was varied across the plate from 1 femtomole to 10 nmoles per well. The TNT was incubated in the wells for 30 min and 1 pmole TNP-G6PDH was added and incubated for 2 hrs. Bound G6PDH activity was measured as usual and found to decrease at TNT concentrations greater than 10 pmole. To detect smaller quantities of TNT, less IgG is needed. Therefore, the whole assay was scaled down 10 to 100 fold.

To determine the TNP-G6PDH conjugate binding capacity of anti-TNT plates, wells were prepared containing 100 ng anti-TNT IgG. Alternate rows of the plate were not treated with anti-TNT in order that the non-specific binding could be determined at each conjugate concentration and subtracted from the total light output. The amount of conjugate added to the wells varied from 1×10^{-15} moles to 1×10^{-10} moles. Plates were incubated at room temperature with gentle shaking for 2 hours, then washed and assayed for bound G6PDH activity as described. Saturation occurred at the 100 picomole conjugate level with approximately 9×10^{-17} moles of TNP-G6PDH bound specifically to the plate. See Fig. XIII. Non-specific binding at this concentration accounted for 50% of the total light output. This experiment was then repeated using a plate treated

Figure XIII. 100 ng Anti-TNT per Well: Binding Capacity for TNP-G6PDH

Conjugate incubated in wells for 2 hours at 25°C with gentle shaking. Unbound conjugate is removed by washing with PBS-Tween as in text. Values shown are due to specific TNP-G6PDH/anti-TNT interactions (Total Light minus Control Light).
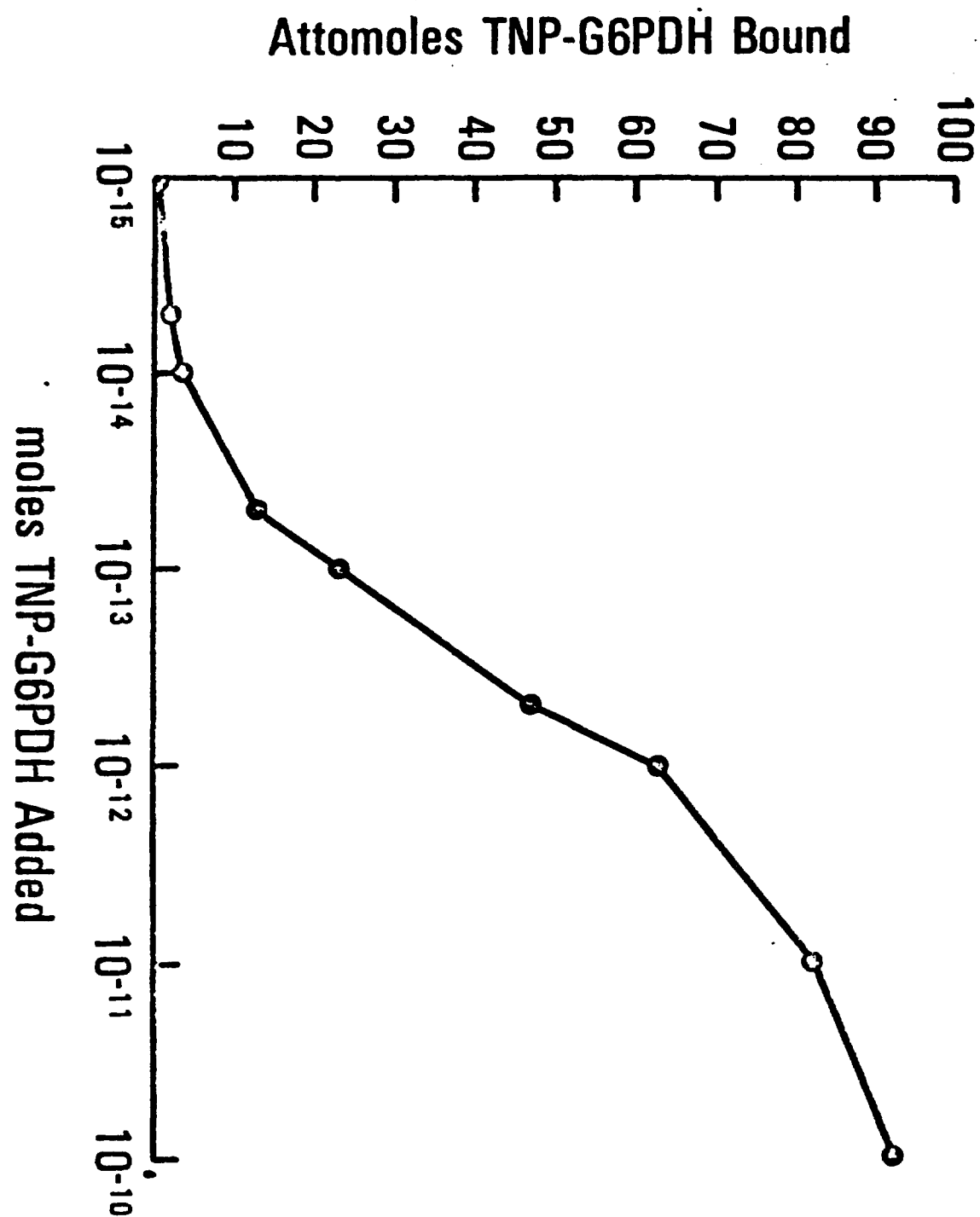
with 10 ng IgG per well. Saturation occurred at 5 picomoles input concentration with a binding capacity of $8.1x10^{-18}$ moles. See Fig.XIV.

Time to Saturation of Immune Plates

Plates were prepared with 10 ng anti-TNT per well. A separate plate was used for each time point in order that subsequent time points would be undisturbed by the washing and assaying protocol. 5 picomoles of TNP-G6PDH conjugate was added to wells at time 0. Non-immune wells were included as blanks. Plates were incubated at room temperature for 30, 60, 120 and 180 minutes, washed and assayed immediately for bound G6PDH activity with the immobilized oxidoreductase-luciferase. Saturation occurred after 60 minutes and remained stable through 180 minutes. See Fig.XV. This is a substantial decrease in incubation time over the 2 hours necessary with Sepharose immobilized antibody system.

Discussion

We have not attempted a competitive assay with free TNT. We need to refine the protocol in order to increase the proportion of light output which is due to specific anti-TNT/TNP-G6PDH interactions. Currently, non-specific binding accounts for 50 to 80% of total bound light producing activity. The coefficient of variation between repetitive samples is in the 5-7% range, which presents problems of quantitative detection when a competitive assay would produce a 10-20% decrease in specifically bound conjugate. We will be further investigating washing protocols and reagents which might reduce this non-specific binding. Agents which might block the binding sites of TNP-G6PDH to the polystyrene will also be tested.

Figure XIV. 10 ng Anti-TNT per Well: Binding Capacity for TNP-G6PDH

Conjugate incubated in wells for 2 hours at 25°C with gentle shaking. Unbound conjugate is removed by washing with PBS-Tween as in text. Values shown are due to specific TNP-G6PDH/anti-TNT interactions (Total Light minus Control Light).
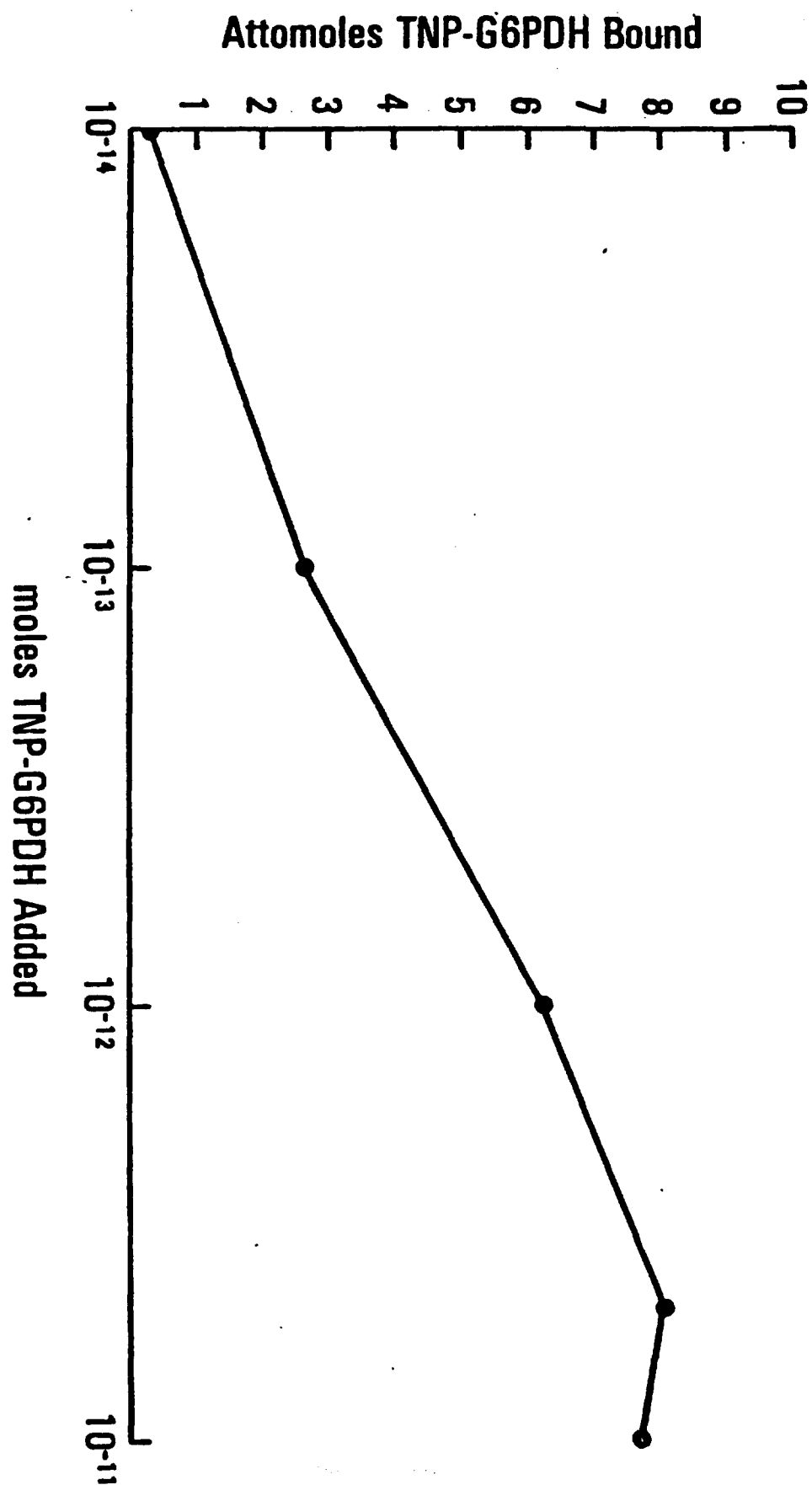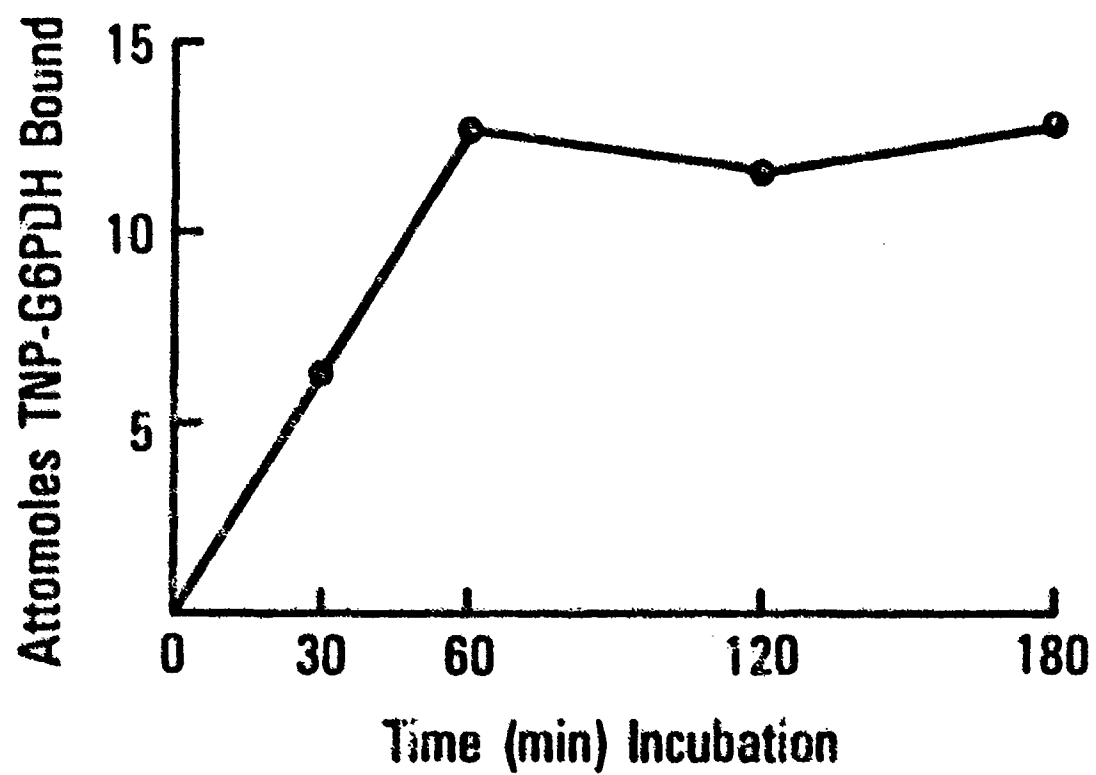
Figure XV.   TNP-G6PDH on 10 ng/well Immune Plate: Time to Saturation

5 picomoles TNP-G6PDH incubated in wells for time shown. Separate plates were run for each time point. Bound conjugate assayed as usual.

f.   Summary of Accomplishments July 1981 - September 1982

1.   Evaluated a procedure using sensitized liposomes as carries for a marker molecule and found it not to be sensitive enough.

2.   Examined some two-phase solvent systems for partitioning free vs. bound TNT. Most were not acceptable, but one system will be re-examined. (See future plans.)

3.   Performed work aimed at the development of a non-equilibrium automated assay, however, the antibody could not be repetitively used in the case of TNT.

4.   Developed a completely automated system with a flow cell which can be used to repetitively measure NADH.

5.   Demonstrated that commercially available diaphorase can replace the NADH:FMN oxidoreductase in the immobilized system.

6.   Developed a protocol for coating microtiter plates with anti-TNP and determined the time course and stoichiometry of binding of TNP-G6PDH to the wells.

g.   Discussion of Accomplishments and Future Plans

Several of the procedures we evaluated do not appear to be viable for any further study: liposomes, or non-equilibrium assay using immobilized anti-TNT.

The two-phase partition experiments, while generally not acceptable, there is one exception which deserves further attention.

Table VI shows that the partitioning of TNP-G6PDH between PEG 8K and $MgSO_4$ exhibits a K/K_{Ab} of 41. It seems to us that this particular system should be evaluated in more detail and some competitive curves with free TNT will be run.

Two very positive aspects are the development of an automated system for measuring NADH and the observation that we can use diaphorase in the immobilized detection system.

With regard to the microtiter plates, we are optimistic about the approach. At the very low levels (10 attomoles) of TNP-G-6-PDH and antibody (femtomoles) we are still plagued with non-specific binding and background problems. However, if these can be overcome, the possibility of automating the entire procedure, i.e., addition of sample, conjugate, washing and adding substrates could easily be automated and this in conjunction with the automated flow system for detecting NADH would then be a very attractive system.

Future Plans

1. We plan to make a TNP-diaphorase conjugate to see whether this might be useful in the development of a homogeneous immunoassay. If this shows promise we will co-immobilize the TNP-diaphorase along with luciferase on to Sepharose. This preparation could then be used in a competitive assay with free TNT. We will start these experiments with Diaphorase since it is commercially available in relatively large quantities. If the sensitivity of this assay is not sufficient (due to a lower turnover number) then it would still be necessary to attempt to clone the NADH:FMN oxidoreductase from B. harveyi so that we have sufficient enzyme to try similar experiments.

Another aspect of this approach is to try to covalently link the diaphorase to the luciferase as a soluble conjugate. This should greatly increase the efficiency of light production for a given amount of NADH, since the enzymes will be in close physical proximity with each other and the product of the oxidoreductase, $FMNH_2$, would be in a local high concentration for utilization by luciferase.

2.   We plan to continue our studies with antibody coated microtiter
plates and using TNP-glucose-6-phosphate dehydrogenase as a label. All of our
experiments to date suggest this approach should be feasible. The major
problem is non-specific binding of the TNP-enzyme to the plates. We plan to
examine a variety of different added proteins, glycerol, PEG's, Triton as well
as other solvents to prevent this non-specific binding. If this is successful
the system could be used in conjunction with the automated flow system developed
by Berthold in which the immobilized oxidoreductase-luciferase would be the
detection system.

3.   We plan to further examine the partition assay utilizing PEG-8K
and $MgSO_4$ which earlier showed some promising results. We will do some
competitive binding studies with TNT and TNP-G-6-PDH in this system to determine
the lower limits of detection.

4.   Finally we plan to purify the TNT-reductase from a strain of soil
bacteria which was previously used by the Beckman group. We have several
affinity columns which we have used for the purification of the NADH:FMN
oxidoreductase and plan to use with the TNT-reductase. The major aim is to
see if we can separate the TNT reductase activity from the NADH oxidase
activity. If we are successful such a system could also be used in an auto-
mated mode with the co-immobilized bioluminescent detection system.

Abbreviations Used

RIA	radioimmunoassay
BIA	bioluminescent immunoassay
LH_2	firefly luciferin
LH_2-AMP	luciferyl-adenylate
G-6-PDH	glucose-6-phosphate dehydrogenase
G-6-P	glucose-6-phosphate
6-PGA	6-phosphogluconic acid
TNP-G-6-PDH	conjugate of TNP and glucose-6-phosphate dehydrogenase
TNP-BSA	conjugate of TNP with bovine serum albumin
TNBS	trinitrobenzene sulfonic acid
TNP-PE	trinitrophenyl-phosphatidyl ethanolamine
PEG	polyethylene glycol
PALA	partition affinity ligand assay
PVP	polyvinyl pyrolidone
TES	N-tris (Hydroxymethyl) methyl-2-aminoethane sulfonic acid
PBS	phosphate buffered saline

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